

**ELECTROCHEMICAL STUDIES OF THE
NICOTINAMIDE ADENINE DINUCLEOTIDE
COENZYME AT PLATINUM , MERCURY AND
MODIFIED GRAPHITE ELECTRODES**



HANS JÄGFELDT

LUND 1982

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by

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Akademisk avhandling som med vederbörligt tillstånd av matematisk-naturvetenskapliga fakulteten vid Universitet i Lund för avläggande av filosofie doktorsexamen kommer att offentligen försvaras å Kemicentrum, Sölvegatan 39, hörsal D, fredagen den 22 oktober 1982, kl 10.15.

Organization University of Lund		Document name Doctoral Dissertation	
Department of Analytical Chemistry		Date of issue October 1982	
Author Hans Jägfeldt		CODEN: LUNKDL/(NKAK-1008/1-129/(1982))	
Title and subtitle Electrochemical Studies of the Nicotinamide Adenine Dinucleotide Coenzyme at Platinum, Mercury and Modified Electrodes		Sponsoring organization:	
Abstract <u>1,4-Dihyronicotinamide adenine dinucleotide (NADH) is determined coulometrically at a rotating platinum gauze electrode. The reaction specificity for the electrochemical oxidation is at least 99.3%.</u> <u>The electrochemical oxidation mechanism of NADH at a platinum electrode is investigated using linear sweep voltammetry. An ECE mechanism followed by dimerization or disproportionation reactions as alternative routes to NAD⁺ is proposed.</u> <u>The products formed in the electrochemical reduction of NAD⁺ at a mercury electrode at -1.1 V and -1.8 V vs SCE are separated by high pressure liquid chromatography (HPLC) isolated and characterized by UV-spectroscopy and NMR.</u> <u>Chemical modification of graphite electrodes by adsorption of mediators at the electrode surface for the electrocatalysis of NADH is described. The modifications have successfully decreased some of the high overvoltage of the NADH oxidation.</u>			
Key words			
Classification system and/or index terms (if any)			
Supplementary bibliographical information			Language English
ISSN and key title			ISBN
Recipient's notes		Number of pages 129	Price
		Security classification	

Distribution by (name and address)

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Electrochemical Studies of the Nicotinamide Adenine Dinucleotide
Coenzyme at Platinum, Mercury and Modified Graphite Electrodes

This thesis summarizes the work presented in the following papers,
referred to in the discussion by the Roman numerals I - V.

- I Electrochemical Oxidation of Reduced Nicotinamide Adenine
Dinucleotide directly and after Reduction in an Enzyme Reactor
H. Jaegfeldt, A. Torstensson and G. Johansson;
Anal. Chim. Acta, 97(1978)221-228
- II Adsorption and Electrochemical Oxidation Behaviour of NADH
at a Clean Platinum Electrode
H. Jaegfeldt; J. Electroanal. Chem., 110(1980)295-302
- III A Study of the Products Formed in the Electrochemical
Reduction of Nicotinamide Adenine Dinucleotide
H. Jaegfeldt; Bioelectrochemistry and Bioenergetics,
8(1981)355-370
- IV Catalytic Oxidation of Reduced Nicotinamide Adenine
Dinucleotide by Graphite Electrodes Modified with Adsorbed
Aromatics Containing Catechol Functionalities
H. Jaegfeldt, A. Torstensson, L. Gorton and G. Johansson;
Anal. Chem. 1982, 53, 1979-1982
- V Electrochemical Stability of Catechols with a Pyrene Side chain
Strongly Adsorbed on Graphite Electrodes for Catalytic Oxidation
of Dihydronicotinamide Adenine Dinucleotide (NADH)
H. Jaegfeldt, T. Kuwana and G. Johansson;
Accepted for publication in J. Am. Chem. Soc.

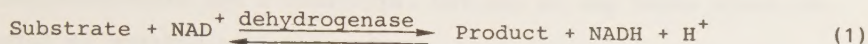
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1. INTRODUCTION

Nicotinamide Adenine Dinucleotide (Fig. 1) is found in all biological systems. It is involved in several hundred known enzymatic reactions [1] such as



where NAD^+ works as a coenzyme to the enzyme dehydrogenase. It is commonly acknowledged that this coenzyme is involved essentially in oxidative biological pathways, such as the reactions in the glycolysis, the Krebs citric acid cycle and the respiratory chain.

These enzymatic reactions are redox reactions in nature, i.e. they involve transport of electrons. They have considerable biological importance, and they have for a long time been of great interest to the electrochemist [2,3]. Also the biochemist could benefit from electrochemical investigations of the pyridine nucleotide. The basic understanding of the electrochemical redox reactions could shed considerable light on the fundamentals of the biological reaction mechanisms. However, of greater interest to the analytical electrochemist is that several quite important applications could be achieved if electrons could be "pumped" easily from an electrode via the coenzyme and a dehydrogenase enzyme to a specific substrate.

The high selectivity of enzymes using NAD^+ as a coenzyme could be exploited in the analysis of complex mixtures with what then could be called substance-selective electrodes [4,5]. Organic synthesis using enzymes as catalysts could be performed without expensive reagents heating energy and waste-problems. Bioelectrochemical cells could be built for transduction of chemical energy into electrical energy using dehydrogenase enzymes as catalysts in the anodic half of a fuel cell [6].

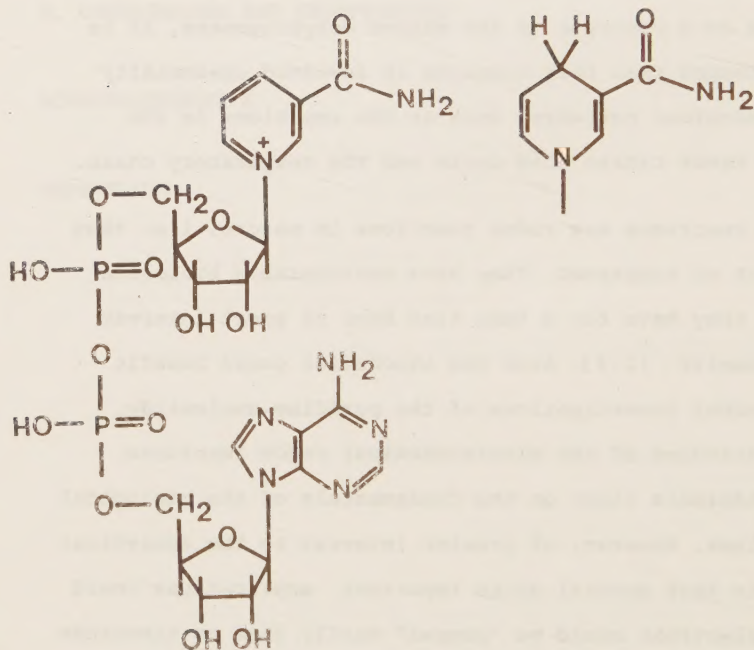


Fig. 1. The structure of NAD⁺ / NADH.

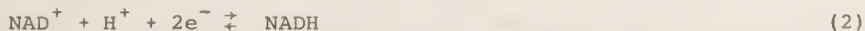
Since "nature does not indulge in luxuries" [7] there are of course basic reasons why the nicotinamide adenine dinucleotide coenzyme reacts so smoothly and reversibly in combination with the dehydrogenases and a substrate, but not at any known electrode material or modification thereof.

Further studies of the electrochemical reactions of the redox couple NAD^+/NADH , the mechanisms, the products and the interactions with electrodes modified with mediators are as a consequence of greatest significance in order to reach the ultimate goal, i.e. a reversible and stable nicotinamide adenine dinucleotide electrode.

2. OXIDATION OF DIHYDRONICOTINAMIDE ADENINE DINUCLEOTIDE

2.1 Normal potential of NADH/NAD^+

The normal potential $E^{\circ'}$ for the overall reaction



has been determined in independent ways. From thermal data and the equilibrium constants for the alcohol dehydrogenase catalyzed ethanol-acetaldehyde and 2-propanol-acetone reactions $E^{\circ'}$ at pH 7.0 (25°C) was calculated to be -0.315 V vs NHE (-0.557 vs SCE) by Clarc [8] and Burton et al. [9]. A direct potentiometric measurement using xanthine oxidase as a catalyst and benzyl viologen as a mediator by Rodkey and Donovan [10,11] yielded $E^{\circ'} = -0.311$ V vs NHE (pH 7.0 and 25°C), in very good agreement with Clarc and Burton.

2.2 Voltammetry of NADH

However, as stated by Burnett and Underwood [12] in an early preliminary work on the oxidation of NADH to NAD^+ , a more positive potential was required than could be attained with a mercury anode.

The oxidation was therefore performed on a platinum electrode, where a poorly defined oxidation wave with a peak in the region of 1 volt was found. In a coulometric experiment run in a pH 6.0 phosphate buffer they arrived at an n value of 2.4, but contended that an enzymatic assay showed quantitative recovery of NAD^+ . The high n value was attributed to a high background current that was not corrected for.

Haas [13] found that NADH was oxidized at 0.9 V vs NHE (0.75 V vs SCE) at a rotating platinum electrode and at 0.67 V (0.42 V vs SCE) at a carbon electrode. LeDuc and Thévenot [14] got results for model compounds on platinum indicating no pH dependence in the range 7-13. This is contrary to reports by Aizawa et al. [15] who obtained a slope $\Delta E/\Delta \text{pH}$ of 35 mV/pH, erroneously claiming that this was in substantial agreement with the Nernst equation not realizing that the E^{O} should move to more negative potentials with increasing pH. Blaedel and Jenkins [16], however, reported $\Delta E/\Delta \text{pH}$ values around -17 mV/pH which is approximately half of what may be calculated from the Nernst equation. From the widely different shifts reported, it must be concluded that they are probably not due to changes in pH, but rather to changes in buffer composition and previous "history" of the electrode itself. The reason for this "indifference" to pH must clearly be the great overvoltage (or high degree of irreversibility) of the oxidation process, i.e. the proton exchange in eq.(2) is not involved in the rate determining step.

2.3 Electrode fouling and number of electrons in the oxidation

Fouling of the electrode, i.e. decrease of peak current and increase of peak potential on successive oxidative scans, has been observed for both platinum and carbon electrodes [13,15-17]. The fouling was attributed to some unknown oxidation products at the electrode surface. For carbon electrodes it was shown that the fouling was caused by adsorption of NAD^+ which is formed in the oxidation of NADH [18]. Elving et al. [19] reported that controlled potential electrolysis at 0.8 V vs SCE at pH 7.0 yielded faradaic n values from 1.3 to 2.0, while cyclic voltammetric data indicated $n = 2$. The report thus indicated that difficulties existed in determining the number of electrons in the oxidation of NADH and therefore also the specificity of the electrochemical reaction in eq.(3).

Aizawa et al. [20] reported, for experiments where NAD^+ was electrochemically regenerated from its reduced form produced in an alcohol-dehydrogenation reaction in an enzyme reactor, that no net loss of the coenzyme could be detected. Their experiments thus indicated high specificity of the reaction in eq.(2). However, from the data given in the report it may be calculated that only ~30% of the total amount of coenzyme was oxidized in the 2 hour experiment. As a consequence the conclusion about high specificity must be considered uncertain.

Since the number of electrons involved in enzymatic reactions with NAD^+ is exactly 2.00 (eq.(1)), a precise knowledge of the number of electrons in the electrochemical oxidation (eq.(3)) is of interest. A value other than $n = 2.00$ would indicate side reactions in the electrochemical process (eq.3)).

2.4 Coulometric oxidation at controlled potential

Basic principles

In controlled potential coulometry the amount of electricity, Q , in a quantitative, stoichiometric electrochemical reaction is used to determine the amount of electrolyzed substance. The amount of substance, X , is correlated to the amount of electricity by Faraday's law

$$Q = n \cdot F \cdot X \quad (5)$$

where F is Faraday's constant and n the number of electrons in the reaction. In the experiment Q is measured by integrating the current electronically

$$Q(t) = \int_0^t i(t) dt \quad (6)$$

2.5 Important "Mass transfer" parameters in the coulometric experiments

The current in a controlled potential coulometric experiment is given by

$$i = n F A D_R \cdot \left(\frac{\partial C_R}{\partial X} \right)_{X=0} \quad (7)$$

where A is the area of the electrode, $(\partial C_R / \partial X)_{X=0}$ the concentration gradient of the reactant at the surface of the electrode. The other parameters have their normal significancies. As convection is used to transport the reactant in the solution to the electrode a simplifying model for the concentration gradient when the potential is applied may be drawn (Fig. 2).

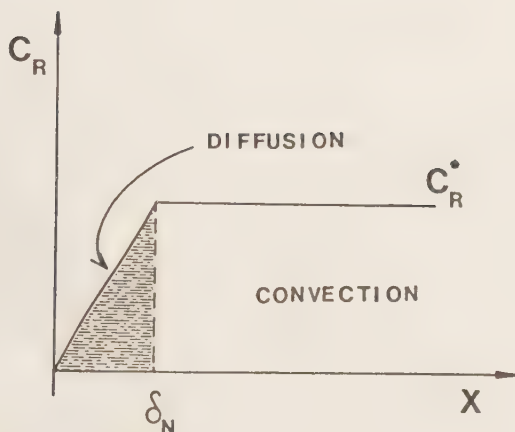


Fig.2. A simplifying model for the concentration gradient when a highly oxidizing potential is applied.

where δ_N is the thickness of the Nernst diffusion layer and C_R^* the bulk concentration of reactant.

As the concentration of the reactant at the electrode surface is zero the current eq.(7) may be written

$$i = n \cdot F \cdot A \cdot D_R \cdot \frac{C_R^*}{\delta_N} \quad (8)$$

The current is also given by the rate of consumption of R

$$i = - n \cdot F \cdot \frac{dN}{dt} \quad (9)$$

where dN/dt is the number of moles consumed per time unit.

As

$$C_R^* = \frac{N}{V} \quad (10)$$

where V is the volume of the solution, we get from eq.(9)

$$i = - n \cdot F \cdot V \cdot \frac{dC_R^*}{dt} \quad (11)$$

From eq. (8) and (11) follows

$$\frac{dC_R^*}{dt} = - \frac{D_R \cdot A}{V \cdot \delta_N} \cdot C_R^* \quad (12)$$

which on integration yields

$$C_R^*(t) = C_R^*(t=0) \exp \left[- \frac{D_R \cdot A}{V \cdot \delta_N} \cdot t \right] \quad (13)$$

which together with eq. (11) yields

$$i = i_0 \exp \left[- \frac{D_R \cdot A}{V \cdot \delta_N} \cdot t \right] \quad (14)$$

$$\text{where } i_0 = \frac{n F D_R A}{\delta_N} \cdot C_R^*(t=0) \quad (15)$$

Equations (14) and (15) show parameters of importance for the rate of the electrolysis, i.e. the parameters for a high current and a fast electrolysis. The electrode area A should be large, the diffusion layer δ_N thin and the volume V of the cell small. These "mass transfer" parameters may be optimized by using a gauze electrode, by dissolving the reactant in a minimum volume of solution and by having a high convection. Efficient convection is best accomplished by rotating the electrode itself, instead of only the solution by using a stirring bar or something equivalent.

When these parameters are taken into account in the coulometric experiment, together with an appropriate correction for background current (Fig. 3), good accuracy and precision may be achieved in determining the amount of electricity measured in the coulometric measurement of NADH (paper I).

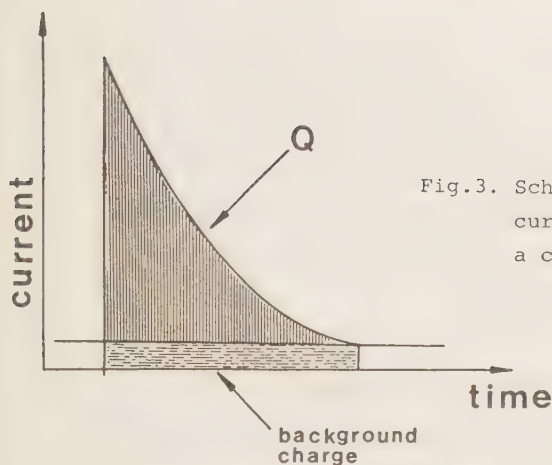


Fig.3. Schematic diagram of the current-time integral in a coulometric experiment.

2.6 Results and conclusions

In the coulometric determinations of NADH described in paper I, it was found that the state of the electrode surface was of crucial importance for obtaining fast kinetics and reproducible results. After the described pretreatment several oxidizing cycles could be performed without appreciable decrease of reaction rate.

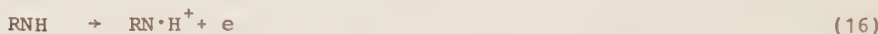
In a number of experiments where 1-3 μmoles NADH were oxidized, current efficiencies of $100 \pm 1\%$ were recorded. These experiments therefore indicated that the electrochemical oxidation of NADH to NAD^+ at a platinum electrode, was quantitative within the available accuracy.

In order to increase the accuracy in the determination of the reaction specificity, the regenerative method described by Aizawa et.al.[21] was used. In these experiments the same batch of NADH was oxidized electrochemically and reduced enzymatically with alcoholdehydrogenase with alcohol up to 24 times. The experiments, one run in a cyclic and another in a continuous mode, showed a

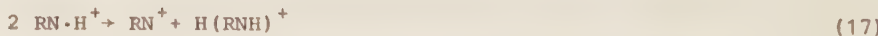
reaction specificity of $\geq 99.3\%$. However, an important observation is also that $< 0.7\%$ of the electrochemically oxidized NADH could not be regenerated enzymatically in each cycle. As the non-electrolytical decomposition was compensated for, the non-regenerative fraction must have been lost in some unknown side reaction or reactions.

3. ELECTROCHEMICAL OXIDATION MECHANISMS OF NADH

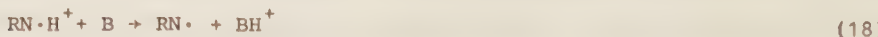
3.1 Blaedel and Haas [22] investigated the electrochemical oxidation of a number of model compounds in acetonitrile at platinum and carbon electrodes in alkaline and unbuffered solutions. They found that the oxidation was resolvable into at least two steps, when no base was added. On the basis of these and other experiments they considered the oxidation of the model compounds to proceed by the reaction



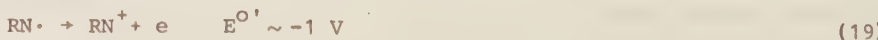
where $\text{RN}\cdot\text{H}^+$ is the protonated pyridinyl radical. In the absence of base, the $\text{RN}\cdot\text{H}^+$ was suggested to disproportionate to form RN^+ according to



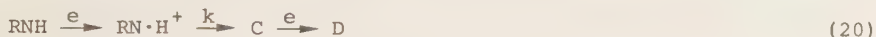
In the presence of base only one peak was observed. The protonated $\text{RN}\cdot\text{H}^+$ radical was proposed to react with the base B according to



where the neutral radical was oxidized immediately to RN^+ through the reaction

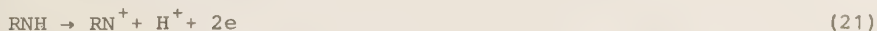


The process responsible for the second peak was not discussed directly by the authors, although it was beautifully shown that the species causing it was produced in an ECE mechanism;



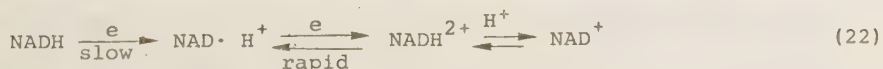
where k is the rate in a chemical step.

As it was found that coulometric oxidation of RNH at wave I quantitatively produced RN^+ the result of a similar experiment past wave II could have yielded important information about the nature of the intermediate species (C). Unfortunately this experiment was not performed. Elving et al. [19] oxidized NMNH (dihydronicotinamide adenine mononucleotide) and NADH in aqueous and DMSO solutions and found only one well defined anodic peak at +0.7 V vs SCE. Added bases showed no effect on the oxidation peak and it was concluded that the best fit in their study was considered to be the direct 2e reaction



However, it was also concluded that poor solubility of NADH and NMNH in acetonitrile prevented a study comparative to the one of Blaedel and Haas. The differences in behaviour might be attributed to differences in basicity of the solvents used (acetonitrile vs DMSO).

LeDuc and Thévenot [14,2] proposed two possible mechanisms for the electrochemical oxidation of model compounds,



and



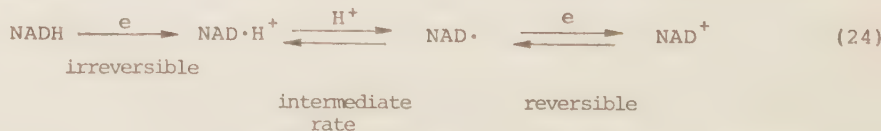
on the basis of voltammetric and rotating ring disc experiments in aqueous solutions.

3.2 Pretreatment of the platinum electrode for the mechanistic study

As mentioned earlier great difficulties in oxidizing NADH clearly and reproducibly at solid electrodes had been encountered. For the mechanistic investigation the pretreatment described in the coulometric experiment (paper I) was found insufficient. In order to get a clean electrode surface, before the test scan in NADH solution was run, a cyclic scanning procedure over a large potential range, analogous to the one described by Blaedel and Haas [22] was used. It was important that the scanning was performed in a buffer solution without NADH. In the scanning procedure small amounts of adsorbed impurities probably desorbed, as was indicated by increasing adsorption peaks of hydrogen [23].

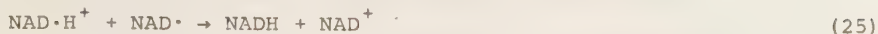
3.3 Conclusions and interpretation

From the linear voltammetric experiments (paper II) the reaction mechanism



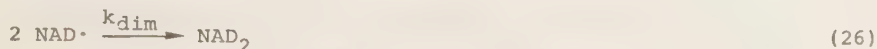
was put forward. However, evidence for a pH dependent second order chemical step was also found. At least two different reaction pathways involving second order chemical reactions all leading ultimately to NAD^+ are possible.

One is the disproportionation reaction



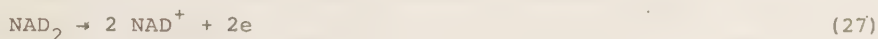
where the formed NADH again could be electrochemically oxidized.

Another is the dimerization reaction



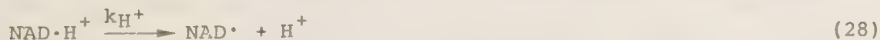
which is a quite fast reaction, $k_{\text{dim}} \sim 3 \cdot 10^6 \text{ M}^{-1}\text{s}^{-1}$ [24].

This chemical step would immediately be followed by an electro-chemical step



as the dimer is oxidizable already at $\sim -100 \text{ mV SCE}$ [3].

The data is not sufficient for deciding which one of these reaction pathways that would predominate under the used experimental conditions. The driving force of reaction (21) would be expected to be high, considering a probable great difference in $E^{\text{O}'}_{\text{NAD}\cdot\text{H}^+/\text{NADH}}$ and $E^{\text{O}'}_{\text{NAD}^+/\text{NAD}\cdot}$. However, neither of the reaction pathways would be expected to show rate dependence on pH. The observed pH-dependence may therefore be attributed to the reaction

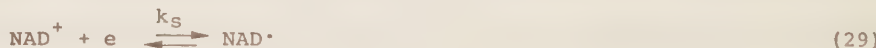


The rate of the second order reaction(s) would thus be indirectly dependent on pH, since the concentration of both $\text{NAD}\cdot\text{H}^+$ and $\text{NAD}\cdot$ close to the electrode would vary with the rate k_{H^+} . The rate constant k_{H^+} will be pH dependent if the reaction in eq.(28) is not completely irreversible.

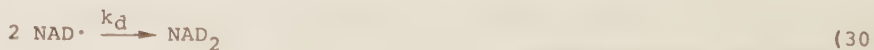
Moiroux and Elving [25] reported almost simultaneously on the mechanistic aspects of the NADH oxidation. They also proposed an ECE mechanism analogous to the one in eq.(24), coupled to a disproportionation reaction between two species $\text{NAD}\cdot\text{H}^+$ and $\text{NAD}\cdot$. They even estimated k_{H^+} in eq.(28) at carbon electrodes to $60 \pm 30 \text{ s}^{-1}$. However, the possibility for dimerization and consequent oxidation to NAD^+ as described in eq.(26) and (27) was not considered.

4. ELECTROCHEMICAL REDUCTION OF NAD^+

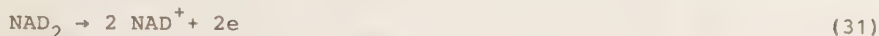
4.1 The electrochemical reduction of NAD^+ and NAD^+ model compounds on mercury electrodes, where the adenosine diphosphate part is replaced by some alkyl or aromatic ring, has been extensively studied [2,3]. It is generally acknowledged that the reduction proceeds in two steps. The first step is the addition of one electron



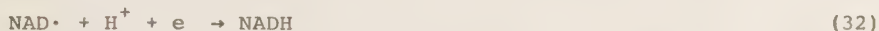
to the nicotinamide ring, yielding the uncharged free radical $\text{NAD}\cdot$ [12]. Polarographically the reaction appears as a wave at approximately -0.9 V vs SCE. The electrochemical kinetics is quite fast [26], but a value for the rate constant $k_s = 0.1 \text{ cm/s}$ was not published until recently [24]. Although the reaction may be considered reversible, it appears quite irreversible even on a cyclic voltammogram with a moderately fast scan rate. This is due to a fast dimerization [2,3,12] reaction



between the formed radicals, $k_d \approx 3 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}$ [24,27]. The dimer is reoxidizable to NAD^+ at approximately -0.1 V vs SCE,



The second reduction step is not as well studied and understood [2]. When adsorption of NAD^+ is depressed by addition of Et_4NCl [28] it appears as an irreversible wave at approximately -1.7 V vs SCE. Studies in aqueous and non-aqueous solutions, with and without proton donors [29], indicate that the reaction proceeds in a stepwise fashion. The radical is first formed according to equation (29), the NADH is then formed by the addition of a proton and an electron simultaneously.



That the radical $\text{NAD}^\bullet$ is involved in the second step is supported by the fact, that some dimer NAD_2 is formed, i.e. some of the free radicals dimerize before the second electron transfer can occur.

Considerable attention has been paid to the structure and composition of the products that are formed in these reactions. However, they have never been isolated and subjected to a detailed structure analysis. The structures of the dimers of NAD^+ , NAD_2 , have been more or less postulated to be the 4,4' or 6,6' dimers [27-30], Fig. 4. However, for some model compounds a thorough investigation of their structures was made [31-33]. The 3-Cyano-1-methyl pyridinium Iodide dimers were found to comprise two pairs of diastereo isomers of the 4,4' and 4,6' compounds [31], while for 1-benzyl-3-carbamoyl pyridinium chloride only a 4,4'-dimer could be isolated and characterized [33]. The NAD_2 species themselves have so far only been analyzed in mixtures [34-36].

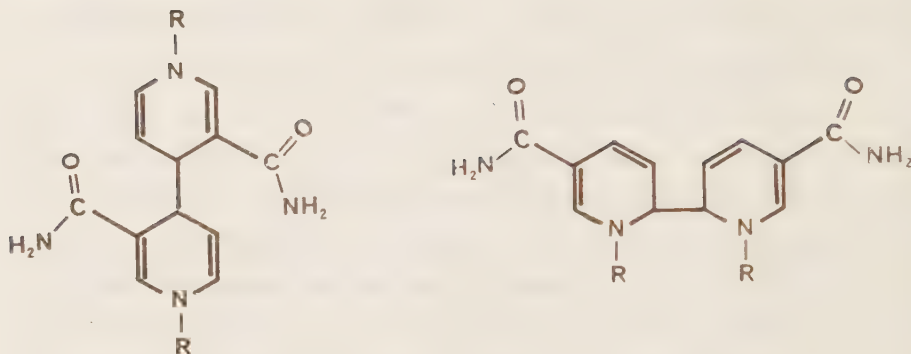


Fig.4. The figure shows the structure of the 4,4' and the 6,6' dimers of NAD⁺, R represents the rest of the NAD⁺ molecule.

This thesis reports on a detailed analysis of the NAD₂ species (paper III). The dimers were separated by gel-filtration and high pressure liquid chromatography (HPLC). They were then collected and subjected to UV-spectroscopic and NMR analysis.

4.2 Conclusions

The results demonstrate the usefulness of the new chromatographic methods in resolving electrochemical problems.

Of course, often shortlived intermediate species are studied that may not be isolated. Other techniques, such as rapid scanning spectrophotometry used in conjunction with optically transparent electrodes may then yield additional important information on the intermediates [37] which otherwise may only be indirectly established in an electrochemical experiment.

5. CHEMICALLY MODIFIED ELECTRODES

5.1 To describe electrodes that have molecules intentionally immobilized on their surfaces, Murray et al. [38] introduced the term chemically modified electrodes. Lane and Hubbard [39] were among the first to use strong adsorption of olefinic compounds on platinum and to study in detail the electrode reactions. Miller et al. [40] coupled optically active amino-acids to graphite. The modified electrode was called a "chiral" electrode and could be used to produce optically active alcohols by the electrochemical reduction of ketones. Anson and coworkers discovered that some organic redox reagents and complexes could be irreversibly bound to graphite electrodes by strong adsorption [41,42], while maintaining excellent redox characteristics.

5.2 Preparation, carbon electrodes

There are several different ways to modify an electrode surface chemically,

- A covalent bonding
- B polymer coating
- C adsorption

A. The covalent bonding method is based on rather simple organic coupling reactions. The first step is usually to introduce oxygen-containing functional groups on the electrode surface. This may be accomplished by several techniques, including air or chemical oxidation anodization and radio-frequency plasma etching [43,44]. The surface oxidized in the form of carboxylic acids may then be transformed to acid-chlorides using e.g. SOCl_2 [45]. This activated surface may be used directly in amidization reactions with some

redox compound, $R-NH_2$. In another method the oxygen functionalities, produced as above, are reduced by some reagent, e.g. $LiAlH_4$, to produce a large number of hydroxyl groups. The hydroxyl groups may then be reacted with a bifunctional molecule, e.g. trichlorotriazine. A surface thus activated will couple to any $R-NH_2$ containing redox compound [46]. This procedure yields modified electrodes with higher coverages compared to the method where the oxidized surface was used directly. The reason is that more oxygen functionalities are accessible to coupling.

B. In the polymer coating method the redox species is incorporated into the backbone of the polymer [47]. The polymer adheres to the electrode surface by some combination of adsorption and low solubility in the contacting solution or by covalent bonding. Polymers have been coated on electrodes by dip and spin coating, organosilane bonding, electrochemical precipitation, polymerization and adsorption from solutions [48].

C. The adsorption method of immobilization uses the ability of many organic compounds to "stick" to a carbon surface. The attraction forces are caused by the high polarizability of this surface and the aromatic rings in an organic compound. It has been found that an increasing number of aromatic rings leads to an increase of the strength of adsorption [41,42]. The lower solubility of these compounds in aqueous solutions also contributes to what will appear as a stronger adsorption. The method of preparation is quite simple and fast. The compound to be adsorbed is dissolved in an appropriate solvent after which the electrode is dipped into the solution. The amount adsorbed is governed by several factors [49,53],

- a) concentration in solution
- b) time spent in the solution
- c) degree of convection
- d) strength of adsorption, ΔG_{Ads}
- e) solubility of the compound in
the contacting solution, ΔG_{Sol}

5.3 Structure of surface layers

The three methods of making CME:s described above, give electrodes with different surface characteristics. The covalent method yields electrodes with monolayer or sub-monolayer coverages, and it is principally not possible to attain higher coverages. This is not an inherent disadvantage, but it rather facilitates a fast electron exchange between the electroactive redox centers and the electrode surface, since the distance between them is very short.

A polymer modification on the contrary may produce both a sub-monolayered or a multi-layered electrode. Depending on the character of the polymer backbone and the chainlengths attained in the polymerization, the distance between the redox centers and the electrode surface may be great even for a low coverage (Fig.5).

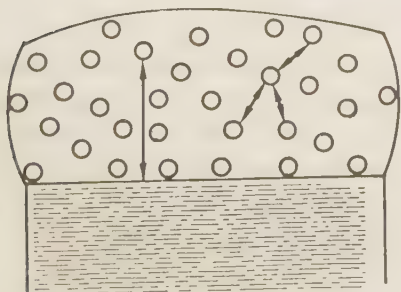


Fig.5. The distance between the redox centers and the electrode may be considerable for a polymer modified electrode. The distance between two individual centers may also be significant.

For an electrode with a very high coverage the propagation of the redox reaction through the polymer layer may be impaired due to this distance [47,50]. In electrocatalysis this may be an undesired effect, as the slow propagation of electrons through the polymer will show up as a shielding of the electrode [51] towards a species in the solution.

With the adsorption method it is also possible to make CME-s with sub-, mono- or multi-layers. The maximum number of layers attainable will depend strongly on the strength of adsorption and the solubility of the adsorbed species in the solution contacting the electrode (paper IV, V). The distance between an adsorbed monolayer and the electrode surface is as short as for the covalently modified electrode. However, contrary to the polymer CME the distance between the electroactive centers do not increase very much if several layers start to build up. The reason is the fact that an adsorbed species does not carry a spacious polymer backbone and a second layer must lie directly on top of the first one. Slow propagation of the redox reactions through several adsorbed layers may therefore not be caused by large distances between the redox centers (Fig. 6).

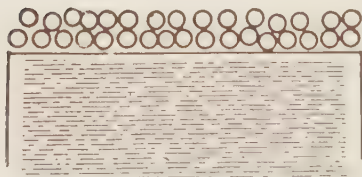


Fig.6. For an adsorbed mediator a second layer will lie directly on top of the first one. The distance between individual mediators will be small as well as the distance to the electrode surface..

If the species immobilized exchange electrons reversibly with the electrode, fast chemical redox reactions between several adsorbed layers are also expected. As a consequence a shielding effect induced by the many layers, as discussed for a polymer coated CME, will not be observed [51].

6. ELECTROCHEMICAL CHARACTERISTICS, ADSORBED SPECIES

6.1 Reversible redox behaviour

Assuming that the adsorption and the electrochemical reactions both are at equilibrium, also assuming that the adsorption sites are equivalent and that there are no interactions between the molecules, i.e. conditions corresponding to a Langmuir adsorption isotherm, the equation for the i-E curve is then given by,

$$i = \frac{n^2 F^2}{RT} \cdot \nu A \Gamma \cdot \frac{\alpha}{(1+\alpha)^2} \quad (33)$$

This equation describes a symmetrically shaped current-potential curve (Fig. 7), where Γ , the total surface concentration is the sum of the ox, Γ_{ox} , and the red form, Γ_R ,

$$\Gamma = \Gamma_O + \Gamma_R \quad (34)$$

and

$$\alpha = \frac{b_O}{b_R} \cdot \exp \frac{nF}{RT} (E - E^{O'}) \quad (35)$$

where b_O and b_R are the adsorption coefficients for the ox and red forms respectively,

$$b_O = \exp(-\Delta G_O/RT) \quad (36)$$

$$b_R = \exp(-\Delta G_R/RT) \quad (37)$$

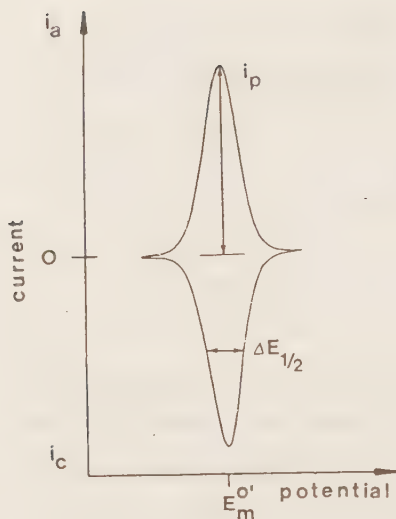


Fig.7. A symmetrically shaped current-potential curve is acquired in a voltametric experiment with an adsorbed species which rapidly exchanges electrons with the electrode.

In eq.(36) and (37) ΔG_O and ΔG_R are the standard free energies of adsorption. All other parameters have their usual significancies.

When $E = E^{O'}$ the current has reached its maximum value

$$i_p = \frac{n^2 F^2}{4RT} \cdot \nu A \Gamma \quad (38)$$

which also from eq.(33), (34) and (35) gives the peak potential

$$E_p = E^{O'} - \frac{RT}{nF} \ln\left(\frac{b_O}{b_R}\right) \quad (39)$$

Equation (39) shows that the peak potential will be equal to or very close to $E^{O'}$ if the energy of adsorption for the two species are the same. It also follows that $\Delta E_p = E_p, \text{ anodic} - E_p, \text{ cathodic} = 0$ and that the width at half peak-height is given by $\Delta E_{p/2} = 90.6/n$ for a reversible redox behaviour [53].

6.2 Factors influencing the shape of the wave

A series of factors may affect the symmetrically shaped voltammetric wave described above.

A. Slow kinetics in the electron exchange between the electrode and the immobilized species will make the peak separation dependent on k_s and α , the heterogeneous rate constant and transfer coefficient, and the used scan rate v . Laviron [54] derived a graphical method by which it was possible to correlate $n \cdot \Delta E_p$ and a factor $1/m$, where

$$m = \left(\frac{RT}{nF} \right) \cdot \frac{k_s}{v} \quad (40)$$

The separation of the peaks increase with increasing scan rate and decreasing rate constant k_s . The transfer coefficient begins to have a significant effect on the shape of the wave for peak separations greater than 50 mV.

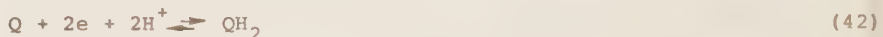
B. Interactions between the immobilized species i.e. Ox-Ox, Red-Red and Ox-Red will affect the shape of the wave. If the interactions are repulsive the voltammogram will become lower and broader. For attractive interactions the peaks will become more narrow and give a higher peak current. The position of the peak will also be influenced by the constants of interaction a_O and a_R .

$$E_p = E^{O'} - \frac{RT}{nF} \ln \left(\frac{b_O}{b_R} \right) - \frac{RT}{nF} (a_R - a_O) \cdot \frac{\Gamma}{\Gamma_{\max}} \quad (41)$$

as well as the relative surface coverage $\Gamma/\Gamma_{\max}$ [55].

C. Slow rates of the chemical redox reactions between redox couples inside a multilayer will broaden the voltammetric peaks and "tailing" will start to appear. This will happen even when the electrochemical reaction at the surface is fast [56]. For very thick layers with slow chemical reactions between the layers, the voltammogram will start to look like one where the current is controlled by diffusion [47,50].

D. It has been observed that a low buffer capacity affects the separation between the oxidation and reduction peaks [52]. An explanation for the observation is that the pH is changed locally at the surface. The depletion or excess of protons is caused by the surface reaction (quinones),



Protons are produced on an anodic scan, therefore the pH close to the electrode will decrease if the buffer capacity is too low. According to the Nernst equation applied to eq.(42)

$$E = E^{O'} - 2.3 \frac{RT}{F} \text{pH} - \frac{RT}{2F} \ln \frac{[QH]}{[Q]} \quad (43)$$

the wave will move to slightly more anodic values. The opposite will happen for a cathodic scan, i.e. a separation of the peaks is induced. The effect is more pronounced for lower buffer capacities and higher surface coverages and for faster scans where diffusion of the buffer will be negligible [52].

A detailed theoretical description of this phenomena has so far not appeared in the literature, and is therefore under way in this laboratory.

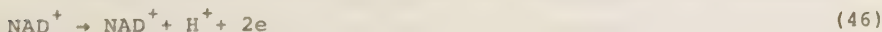
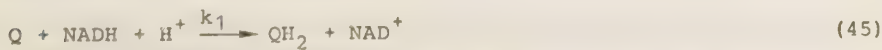
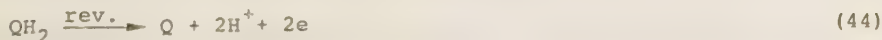
7. HETEROGENEOUS ELECTROCATALYSIS OF NADH WITH MODIFIED ELECTRODES

7.1 Tse and Kuwana [57] were the first who showed that it was possible to make a CME which catalyzed the NADH oxidation. The catalyst they used, 3,4-dihydroxybenzylamine, had earlier been found to oxidize the NADH readily in solution. They used the covalent method of immobilization discussed above in order to couple the catalyst to the surface. Degrand and Miller [59] utilized the polymer coating method for the same purpose. They incorporated dopamine into the back-bone of poly-methacryloyl chloride, which then was attached to the electrode surface. This electrode also showed catalytic effect. However, none of the electrodes retained catalytic effect for more than a few cycles.

It was found in this laboratory, that adsorption was a very powerful technique to produce CME:s with high and easily variable coverages [60]. Therefore work was undertaken to find out whether the adsorption method also could be used as a means to give catalytic electrodes (paper IV). The instability and deactivation processes for the quinone catalyst observed both by Kuwana et al. and Miller et al., were studied in paper V.

7.2 Theory

The catalytic electrodes discussed above are all made by attaching a redox couple which has a fast electron exchange with the electrode. The oxidized form of the redox couple also reacts chemically with NADH with the rate k_1 . The purpose of the redox couple is to work as a mediator in an EC-catalytic process



As the objective is to decrease overvoltage, it is of importance that the $E_m^{O'}$ of the mediator is as close as possible to the reversible $E_{NAD^+}^{O'}/NADH$ for the coenzyme. It is also important that the rate of the chemical reaction is not too low, $k_1 > 1 \cdot 10^3 M^{-1} s^{-1}$. In principle a catalytic process is possible, if no activation energy barriers are present in the chemical step eq.(45), even when $E_m^{O'}$ is slightly lower than $E_{NAD^+}^{O'}/NADH$. However, the equilibrium of the reaction will quickly become unfavourable.

Tse and Kuwana [57], who made stop flow measurements for a series of quinones, found that the reaction rate constant, k_1 , in eq.(45) was a function of $E_m^{O'}$, and consequently of $\Delta G_m^{O'}$. For a homogeneous reaction involving energy barriers a behaviour like this is expected, since it is reasonable to assume that the barriers within such a "homologous" series of compounds should be of relatively constant magnitude.

Andrieux and Savéant [61] derived an expression for the catalytic peak current in cyclic voltammetry

$$i_p = 0.496 \cdot nFA \cdot (D_R \frac{nF}{RT} v)^{\frac{1}{2}} \cdot C_R \quad (47)$$

where the constant 0.496 (max value) was found to vary with the function

$$\lg[k_1 \cdot \Gamma / (D_R nFv/RT)^{\frac{1}{2}}] \quad (48)$$

For a given scan rate v and coverage Γ , the rate of the reaction, k_1 , in eq.(45) becomes very important for a high catalytic current. A decrease of k_1 from $1 \cdot 10^3 M^{-1} s^{-1}$ to $1 \cdot 10^1 M^{-1} s^{-1}$ ($\Gamma \approx 1 \cdot 10^{-9} \text{ mole/cm}^2$, $v = 50 \text{ mV/s}$) would result in an almost complete loss of observable catalysis. However, a more sensitive and accurate way to detect catalysis and to measure the rate constant k_1 , is to use the rotating disc electrode [62]. When the catalytic current reaches its maximum value it is given by

$$i_{\text{cat,max}} = nFA \cdot k_1 \cdot \Gamma \cdot C_R \quad (49)$$

The reason for the higher sensitivity and accuracy is that the magnitude of the current in this case only is governed by the reaction rate constant k_1 . If $k_1 = 0$ the current from the oxidation of the mediator itself will decrease to zero within seconds. One might say that the mechanism of the catalysis changes from an EC scheme in cyclic voltammetry to a CE one when using the rotating disc electrode.

Another method to measure the rate constant k_1 was given by Savéant et al. [63]. In this case the rate constant is measured at one rotational speed with the RDE by comparing the limiting catalytic current i_{cat} with the limiting current for the uncatalyzed reaction i_1 (Fig. 8).

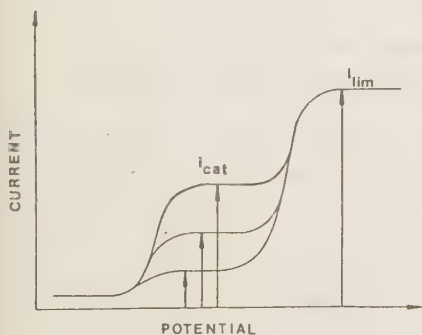


Fig.8. The rate constant k_1 in eq. (45) may be calculated by comparing the uncatalyzed limiting current, i_{lim} , with the catalytic limiting current, i_{cat} .

The rate constant may be calculated from the equation

$$k_1 = \frac{i_C / \delta}{(\Gamma / D_R) (i_1 - i_C)} \quad (50)$$

where

$$\delta = 1.61 \cdot \nu^{1/6} D_R^{1/3} \cdot \omega^{-1/2} \quad (51)$$

D_R is the diffusion constant for the reactant, ν the kinematic viscosity and ω the rate of rotation. The other parameters have their usual significancies.

7.3 Other mediators than ortho-quinones

Torstensson and Gorton [64] showed that N-methylphenazinium (PMS) and N-ethylphenazinium (PES) both catalyzed the NADH oxidation very rapidly [64]. The rate of reaction with NADH, $k_1 \approx 5 \cdot 10^3 \text{ M}^{-1} \text{ s}^{-1}$, was as fast as the one for the ortho-quinones (paper V). Still the $E_{\text{pH}=7}^{\text{O}'}$ = -160 mV (vs SCE) for PMS is much more negative than that for the ortho-quinones $E_{\text{Orth.}}^{\text{O}'} \approx +200 \text{ mV}$. Obviously the activation energy barriers are much lower for the PMS. Almost simultaneously Miller et al. in a study of 25 different mediators (in solution) found that "quinone" diimines showed to be very effective in the NADH catalysis. They demonstrated that 1,2-diimines had a somewhat higher rate of reaction than 1,4-diimines. Basically PMS may be regarded as a 1,2-diimine when it is in the oxidized stage. More recently Gorton et al. [52] found that Meldola blue (Fig. 9) was an excellent catalyst for the NADH oxidation. Meldola blue which is basically a 1,4-diamine adsorbed much stronger than PMS, as the aromatic ring structure is larger. The $E_{\text{pH}=7}^{\text{O}'}$ = -170 mV and $k_1 \approx 6 \cdot 10^3 \text{ M}^{-1} \text{ s}^{-1}$ for Meldola blue were approximately the same as for PMS, but the stability of the Meldola modified electrode in the presence of NADH was quite amazing compared to any other known CME. The very fast chemical step and the impressive stability of the Meldola blue catalysis led us to further investigate some other oxazines that have even lower $E^{\text{O}'}$ values [65]. Fig. 9 shows a series of compounds that have so far been tested. According to Miller et al. [66] the rate of reaction should not be a function of $E_{\text{m}}^{\text{O}'}$. However, our recent results indicate that this is not true, at least when the mediators are immobilized on the electrode surface. Rather, there seems to be a linear relationship between $\lg k_1$ and $E_{\text{m}}^{\text{O}'}$ (Fig. 10), which as stated above, would be expected for a series of mediators where the basic

reaction mechanism is the same and therefore also the magnitude of the activation energy barriers.

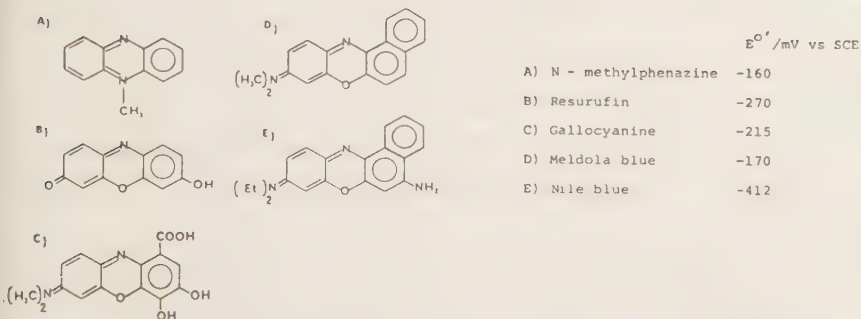


Fig.9. Five mediators that have been immobilized by adsorption and tested for catalytic activity.

8. CONCLUSIONS AND PERSPECTIVES

In the search of effective mediators for the heterogeneous catalytic electrochemical oxidation of NADH, three classes of compounds have been discovered. They are the quinones [57,58,59], the azines [64] and the oxazines [52,65].

The quinones were the first ones investigated[57,58]. However, as it has been found that a coupling reaction between the quinones and NADH gradually blocked the electrode (paper V), this class of mediators will probably not yield the possibility to produce an efficient catalytic electrode.

The azines, although not yet thoroughly investigated, also seemed to give stability problems. Whether the deactivation in this case is caused by coupling reactions to NADH, by other chemical reactions or desorption is not known.

So far the oxazines seem to be the best candidates for producing a good catalytic electrode. A great many oxazines are commercially available [67], many with quite low $E_m^{O'}$ values. The possibility of synthesizing other oxazines with $E_m^{O'}$ values close to $E_{NAD^+}^{O'}/NADH$ should be good. Their chemical stability is excellent at pHs below 8, above where slow hydrolysis leads to ring opening at the oxygen position [68]. The stability of an oxazine modified electrode is very good. However, a very slow decrease in the amount of electrochemically active mediator at the surface of the electrode has been observed before and after NADH catalysis. Contrary to the quinone mediator this loss does not seem to be connected with blocking of the electrode surface.

Unfortunately, the requirement for a fast chemical reaction rate between NADH and the mediator may be difficult to fulfill also with the oxazines. An extrapolation of the data in Fig. 9 yields a rate constant k_1 of $\sim 1 \text{ M}^{-1}\text{s}^{-1}$ for a species with $E_m^{O'} = E_{NAD^+}^{O'}/NADH$. This is three orders of magnitude smaller than needed to see the catalytic effect well using cyclic voltammetry. Further work will show whether the reaction rate is faster than expected from the extrapolation in Fig. 10.

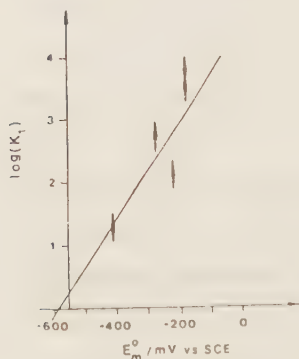


Fig.10. A linear relationship between the logarithm of the rate constant, $\log(k_1)$, for the chemical step (eq.(45)) and the normal potential, $E_m^{O'}$, for the mediator was found.

ACKNOWLEDGEMENTS

I want to express my sincere thanks to professor Gillis Johansson, who introduced me to the field of electrochemistry of coenzymes and who has given me continuous support in my work. I also want to express my gratitude to all my friends and colleagues at Analytical Chemistry at the University of Lund, for creating a friendly and stimulating atmosphere. Especially I want to thank Drs. Arne Torstensson, Lo Gorton and Lars Ögren together with Mr Hans Lundbäck and Mr Istvan Csiky for very inspiring discussions and valuable collaboration over the years in Lund. In addition I wish to express my sincere gratitude to professor Theodore Kuwana for giving me the opportunity to visit him and his research group at the Ohio State University, Columbus, Ohio, USA, during 1981. Together with Dr. Kuwana I especially want to thank Drs. Paul Forshey, Dennis DiMarco and Chihiro Ueda for valuable discussions and ideas. Financial support from Kemiska Institutionen i Lund, Knut and Alice Wallenbergs Stiftelse, Stiftelsen Bengt Lundquists Minne and the National Institutes of Health is gratefully acknowledged. Finally I want to thank Elsevier Scientific Publishing Company and the American Chemical Society for permission to reproduce the papers originally appearing in *Analytica Chimica Acta*, *Journals of Electroanalytical Chemistry*, *Bioelectrochemistry* and *Bioenergetics* and *Analytical Chemistry*.

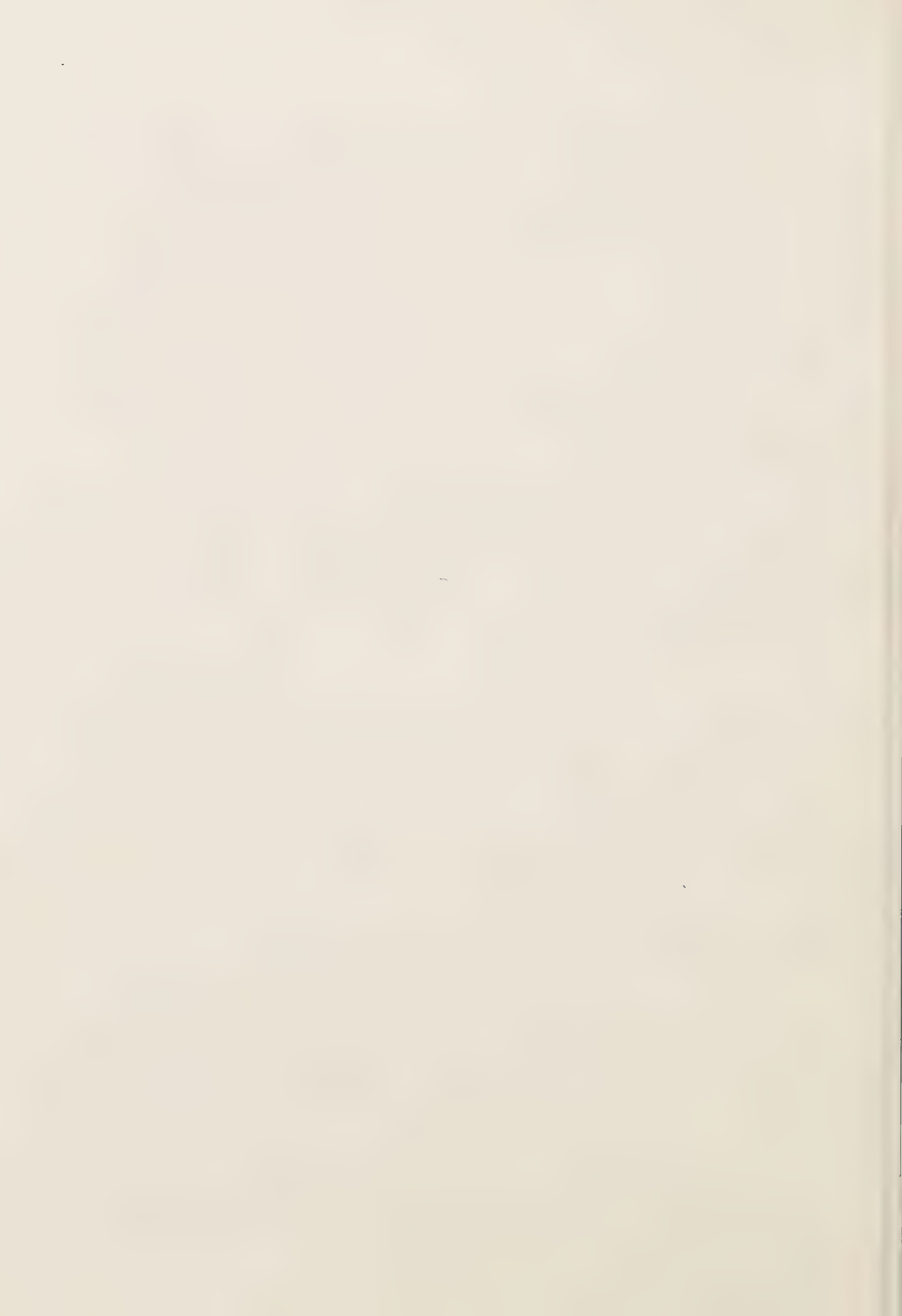
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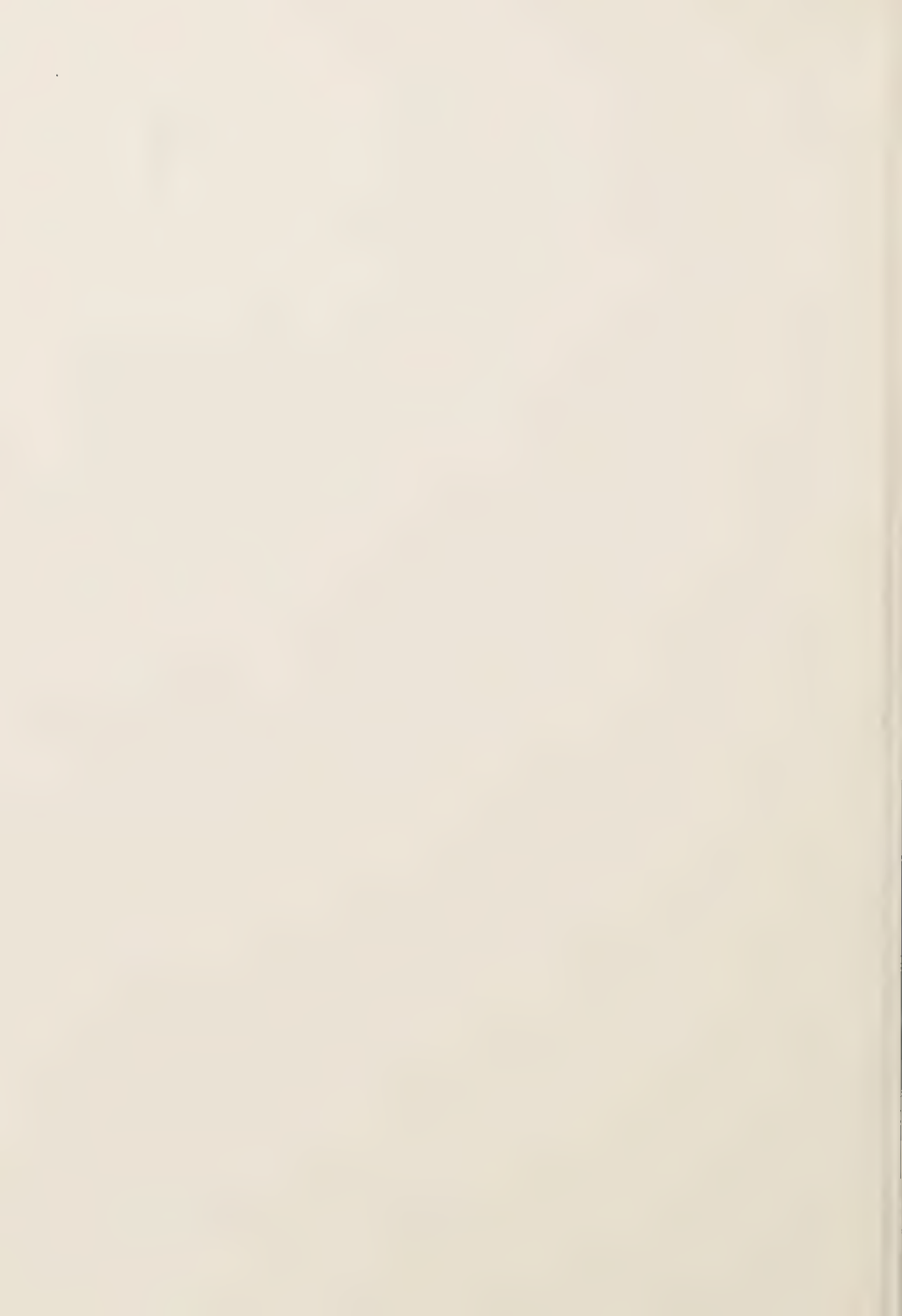
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I



ELECTROCHEMICAL OXIDATION OF REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE DIRECTLY AND AFTER REDUCTION IN AN ENZYME REACTOR

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(Received 5th October 1977)

SUMMARY

Reduced nicotinamide adenine dinucleotide (NADH) was oxidized coulometrically at a rotating platinum gauze electrode at 0.7 V vs. SCE at pH 9. The NAD⁺ formed was reduced enzymatically in a reactor containing immobilized alcohol dehydrogenase. Several recyclings were made with the same lot of NADH. The coulometric results were in reasonable agreement with spectrophotometric measurements. The overall conversion to enzymatically active NAD⁺ showed a current efficiency of 99.3%. The electrode pretreatment described is essential for high current efficiency. A continuous method of oxidation of alcohol with electrolytic regeneration of cofactor and removal of aldehyde by dialysis was tested.

Regeneration of cofactors is of some importance both in enzyme technology and for analytical purposes. Two enzymatic reactions, one for the reduction and one for the oxidation of the cofactors, can be coupled to facilitate regeneration of expensive cofactors. For technical purposes, the disadvantage of this method is that the product is mixed with the reagents and products for the regeneration cycle. For analytical purposes, the regenerating system must be chosen so that selective detection is possible, and the equilibrium conditions must be favourable for the desired reaction. An electrolytic regeneration should have advantages in both applications.

Preliminary experiments by Burnett and Underwood [1] showed quantitative recovery of nicotinamide adenine dinucleotide (NAD⁺) upon electrolytic oxidation of NADH. However, it was found later [2] that the electrode surface became fouled by accumulation of unknown oxidation products. Aizawa et al. [3] studied the voltammetric properties at platinum electrodes and also performed coulometric oxidations at 0.7 V vs. SCE. The electrolytic cell was also incorporated into a continuous-flow reactor system [4]. A slow adsorption was reported and the current efficiency was 95% [4] for reoxidation. The voltammetric and coulometric oxidation of NADH and other cofactors at glassy carbon electrodes was studied by Braun et al. [5]. An n value of 1.5 was found with controlled potential coulometry

(which corresponds to 75% current efficiency), in contrast to the value of 2.0 found from voltammetric measurements. Blaedel and Jenkins [6, 7] used steady-state voltammetry to study the conditions for direct amperometric determination of NADH in micromolar concentrations. This method was selected as it had been found impossible to oxidize NADH cleanly in aqueous systems by conventional voltammetry. It was concluded [7] that platinum was not quite as suitable as glassy carbon for studies of NADH oxidation; it was also shown that even small amounts of Tris buffer blocked the electron transfer. Thomas and Christian [8] devised an amperometric method for alcohol determination using electrochemical oxidation of NADH. Wallace et al. [9] used NADH oxidation for enzyme determinations in a flow system. Blaedel and Jenkins [10] described a lactate electrode in which the NADH was regenerated electrochemically.

In most of the references cited some difficulties have been encountered in the oxidation, and various methods of electrode pretreatment have been devised. The reasons for the lack of electrode reproducibility are not well understood although adsorption may be of some importance. Why a given pretreatment should change the adsorption behaviour is not easy to understand.

The present investigation is a continuation of the investigation by Aizawa et al. [3] and the condition of the electrolysis is similar. In addition to the spectrophotometric method used by these workers, a coulometric method capable of high precision is included in the present study. The continuous regeneration unit also follows a design described earlier [4] but the conditions used in this work allowed a simultaneous and precise coulometric measurement.

EXPERIMENTAL

Electrochemical oxidation of NADH was done in a three-compartment cell as shown in Fig. 1. Sample was added to the working electrode compartment which contained a rotating platinum-gauze electrode; the gauze was 36 mesh and the electrode diameter was 35 mm, the rotating speed was 550 r.p.m. A mercury contact was provided at the top of the axis. A calomel reference electrode (Radiometer K 401) was placed as close as possible to the rotating electrode. The volume of the chamber was about 20 ml but usually only 15 ml of solution was dispensed into it. Electrolytic connection to the auxiliary electrode was made through two clay-filters with an intermediate chamber in between. The cell was connected to a three-electrode potentiostat with electronic integration and direct read-out of the current-time integral on a digital voltmeter.

The electrolyte was either 0.1 M or 0.05 M sodium pyrophosphate which was adjusted to pH 9.00 with 5 M HCl. Various amounts of NADH (Sigma Chemicals Company, Cat. No. N-8129) were added to the solution in the working electrode compartment. In some experiments ethanol was present and in this case an ethanolic buffer was used in all three compartments.

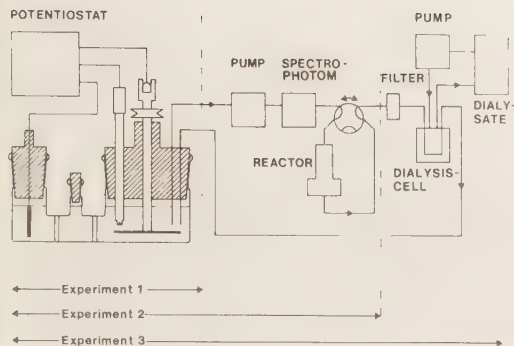


Fig. 1. Diagram of experimental arrangement showing which parts were used during the three reported reactions.

Figure 1 shows the experimental arrangement. The first series of coulometric oxidations was made in the cell, the left part of Fig. 1, with no external devices connected. In the next series of experiments, an enzyme reactor containing immobilized alcohol dehydrogenase was added in an external loop. A peristaltic pump (LKB 12000 Varioperpex) pumped the solution at a flow rate of 5 ml min^{-1} . A flow-through 10-mm spectrophotometric cell was also inserted into the loop. The spectrophotometer was a Zeiss PMQ II. The enzyme reactor could be bypassed by switching a sample loop (Altex Rotary Valves, Series 202) which had a volume of 2.7 ml. The third arrangement included a hollow-fiber dialysis cell (Bio-Rad Laboratories, model Bio-Fiber 20 Beaker) with a cut-off at 200D. The dialysate consisted of 1100 ml of exactly the same buffer solution as that filled into the loop and electrolysis cell except that no NADH was present in the dialysing solution. The solution was pumped through the hollow-fiber unit at 10 ml min^{-1} . The volume of the external cell loop was increased by about 4 ml when the dialysis cell was inserted. The loop also contained a Millipore filter unit (Swinnex-13 model) with a $5\text{-}\mu\text{m}$ cellulose acetate filter to prevent clogging of the hollow fibers.

The alcohol dehydrogenase (Sigma Chemical Company, Cat. No. A-7011) was immobilized on porous glass (CPG-10/700 Å, 80–120 mesh, pore diameter 654 Å; Serva Feinbiochemica) with glutaraldehyde as described earlier [11]. The immobilized enzyme was filled into a reactor (i.d. 5 mm, length 55 mm) and held with $5\text{-}\mu\text{m}$ Millipore filter discs at the lower end. The porous glass was not an ideal support and the reactor became deactivated several times. Alumina would probably have been a better support [4].

RESULTS

Single-pass electrolysis

NADH was dissolved in 0.1 M sodium pyrophosphate to give a stock solution of about 0.15 mM. A more exact determination of the concentration was made spectrophotometrically, based on a molar absorptivity of 6220 $\text{l mol}^{-1} \text{cm}^{-1}$ [12]. An aliquot of the stock solution was added to the coulometric cell and oxidized at 0.70 V vs. SCE. The results are shown in Table 1. Some experiments were also made in which a smaller aliquot was taken; the volume was made up with buffer. It can be seen that the coulometric and the spectrophotometric determinations give the same result within 1.3% or better. This shows that a coulometric oxidation of NADH can be made quantitatively, in contrast to some earlier reports [1, 4, 5].

In order to obtain these results, it was necessary to pretreat the working electrode by first washing it in warm, concentrated nitric acid for about 10 min, then rinsing thoroughly in water, and preelectrolysing in 0.1 M pyrophosphate buffer at 0.7 V until the residual current decreased to a value of 10–20 μA . The pretreatment changed the oxidation behaviour drastically. The electrolysis current in both cases followed the equation

$$i_t = i_0 e^{-\beta \cdot t} \quad (1)$$

in which i_t is the current at the time t , e the natural logarithm base, and β and i_0 are constants. After preelectrolysis, values of β were around $8 \times 10^{-3} \text{s}^{-1}$, but without the pretreatment the value of β varied, often being much lower, down to $1 \times 10^{-3} \text{s}^{-1}$. The determinations made after pretreatment indicated 100% current efficiency, i.e. the amount of NADH calculated from the current integral was equal to the amount determined spectrophotometrically. Without pretreatment the current efficiency varied; values of 65%, 116%, 110% as well as some around 100% were obtained.

TABLE 1

Comparison of spectrophotometric (340 nm, $\epsilon = 6220 \text{l mol}^{-1} \text{cm}^{-1}$) and coulometric (Pt, pH 9.0 pyrophosphate, +0.70 V vs. SCE) determination of NADH

NADH (μmol)		Number of runs	Difference $\Delta\%$
Spectrophotometric	Coulometric		
0.79	0.78	1	1.3
0.83	0.82	1	1.2
1.57	1.58	4	-0.6
1.61	1.61	3	0
1.63	1.63	2	0
1.65	1.65	3	0
2.47	2.45	2	0.8

Electrolysis and enzymatic regeneration

For a successful electrolytic oxidation of a cofactor the oxidation product must necessarily be enzymatically active. Alcohol dehydrogenase is dependent on NAD^+ for the reaction



By adding immobilized ADH in a side loop it should be possible to regenerate NADH quantitatively from NAD^+ . Oxidation products other than NAD^+ should not be reduced and it should therefore be possible to achieve a quantitative measure of the inactive side products. In order to increase the accuracy of the measurement, the set-up was arranged so that the same lot of NADH could be electrolytically oxidized, enzymatically reduced, electrolytically oxidized etc. Figure 2 shows the results obtained during such a recycling when the original concentration of ethanol was 0.53 M in 0.1 M pyrophosphate, pH 9.0. The circles give the amount of NADH oxidized as a percentage of the amount ($107 \mu\text{mol}$) originally present.

There is a steady decrease of the amount of NADH which is regenerated. It is known that NAD^+ is unstable in alkaline solution whereas NADH is more stable. The rate of this non-electrolytic decomposition was measured separately by storing a solution of buffer- NAD^+ at pH 9.0 (see below). A complete regeneration of NADH will also be prevented if the acetaldehyde concentration increases. The equilibrium constant of eqn. (2) is known [4]; it was taken to be 2.31×10^{-2} when the H^+ concentration is included. The concentration of NADH after the i th oxidation will then be

$$[\text{NADH}]_i = -X + (X^2 + Y)^{\frac{1}{2}} \quad (3)$$

$$X = ([\text{EtOH}]_i + [\text{NAD}^+] + [\text{CH}_3\text{CHO}]_i / 2.31 \times 10^{-2}) / 84.6$$

$$Y = [\text{EtOH}]_i [\text{NAD}^+]_i / 42.3$$

The acetaldehyde present as an impurity in the alcohol was estimated to be $80 \mu\text{M}$ at the start; the amount was determined by gas chromatography (Varian Aerograph 2700, FID, 4-m column, i.d. 2 mm, 15% PEG on Chromosorb, 65°C). Equation (3) was evaluated by computer with the $80 \mu\text{M}$ start concentration of aldehyde and the result is given in Fig. 2, curve A. Obviously equilibrium considerations alone cannot explain the experimental results.

The non-electrolytic decomposition of NAD^+ , measured spectrophotometrically, as above, was found to be $6.1 \times 10^{-5} \text{ min}^{-1}$. Each cycle took 52 min and therefore the decomposition can be approximated by the equation

$$[\text{NAD}^+]_i = [\text{NAD}^+]_0 (1 - ki) \quad (4)$$

where $k = 1.6 \times 10^{-3}$. Curve B, Fig. 2, shows the result when both the non-electrolytic decomposition and the equilibrium were taken into account.

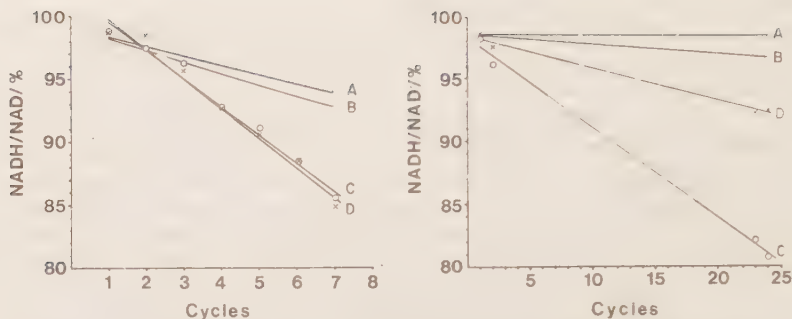


Fig. 2. Recovery of NADH vs. the number of oxidation cycles. Experiment 2. (A) Calculated from equilibrium data. (B) As for A but including pH-dependent non-electrolytic decomposition of NAD^+ . (C) Measured coulometrically (—). (D) Measured spectrophotometrically (x).

Fig. 3. Recovery of NADH vs. number of reoxidations when dialysis was used. Experiment 3. Legends to curves A–D as in Fig. 2.

Curves C and D represent the least-squares lines through the experimental points. The difference between curves B and C and line D is thus a measure of decomposition at the electrode which is estimated to be 1% per cycle.

Recycling with dialysis

To reduce the concentration of acetaldehyde to such a low value that eqn. (2) can be considered to proceed almost completely to the right, a dialysis cell was included in the loop (Fig. 1). The products which could pass the hollow fibers were diluted to a total volume of 1100 ml as compared to the 17 ml in the electrolysis cell, the loop and the hollow fiber unit. The acetaldehyde was thus diluted 65 times compared to the experiment reported above. Some low-molecular-weight products, probably decomposition products from the cofactor, also passed into the dialysate. It is not known if those affect the oxidation. The external solution was periodically checked for the presence of NAD^+ and NADH with a spectrophotometer (McPherson model EU-707-11). No NAD^+ or NADH could be found with the method which had a detection limit of $0.08 \mu\text{mol}$ corresponding to a 6% loss during a run of 9 h 22 min. The sensitivity of the test was thus too low to detect minor losses.

Figure 3 shows the results obtained when $1.34 \mu\text{mol}$ of NADH was dissolved in 0.05 M pyrophosphate buffer, pH 9.0, containing 0.53 M ethanol and $80 \mu\text{M}$ acetaldehyde as an impurity. The total volume was 17 ml. First a complete oxidation was performed with the enzyme reactor bypassed (cycle 1). The enzyme reactor was then used to reduce the NAD^+ completely with the potentiostat switched to stand-by, and the oxidation cycle was then repeated once more (cycle 2). Then the valve was switched

to allow continuous operation. The current integral was taken as a measure of the number of recyclings. After about twenty passes the system was switched back to discontinuous operation to obtain the amount of active cofactor.

Curve C in Fig. 3 is drawn through the points obtained by coulometry and curve D through those obtained spectrophotometrically. Curve A gives the amount expected when the equilibrium constant is taken into account as described above. The line is practically parallel (0.2%/24 cycles) to the abscissa but less than 100% because of the acetaldehyde impurity. Curve B includes also the non-electrolytic decomposition of NAD^+ at pH 9, obtained as above but with a mean cycle time of 23 min during the time of continuous operation.

The two methods give different values. The reason for this lies probably in the different modes of operation. The spectrophotometric method measures a concentration whereas the coulometric method primarily measures an amount (of electricity). Any decrease in volume, e.g. by reverse osmosis through the hollow fibers, could give such a discrepancy with the coulometric method producing the lower value. It was difficult to measure the volume precisely.

The overall result is that NADH can be oxidized electrolytically to enzymatically active NAD^+ with an overall efficiency of 99.3%. This value is obtained when the results from the coulometric method (curve C, Fig. 3) are used.

DISCUSSION

The present results indicate that a quantitative reoxidation of NADH to NAD^+ is possible with a very high current efficiency in a two-electron process; and the product is enzymatically active. These results contrast with some earlier reports in which substantially lower current efficiency was found. Electrode fouling has also been reported [2, 9] as well as adsorption and different behaviour on successive sweeps in voltammetry [3]. The oxidation peak in voltammetry appears at +0.7 V but the half-wave potential for reduction occurs in two steps, 0.9 and 1.7 V [1] at the DME. This indicates a complex electrochemical behaviour.

The pretreatment of the platinum electrode was absolutely essential in order to obtain reproducible and accurate results. The condition of the electrode surface must therefore be an essential factor for the quantitative oxidation of NADH. Probably the condition of the surface affects the adsorption behaviour but the exact mechanism has not been studied further. Another factor which might influence the result is the mass transfer at the working electrode. In the cell used, the platinum gauze was rotated at high speed. A violent turbulence is created around each wire in the mesh and the Nernst diffusion layer is therefore very thin. Any byproducts from the oxidation will thus be transferred into the bulk of the solution very rapidly.

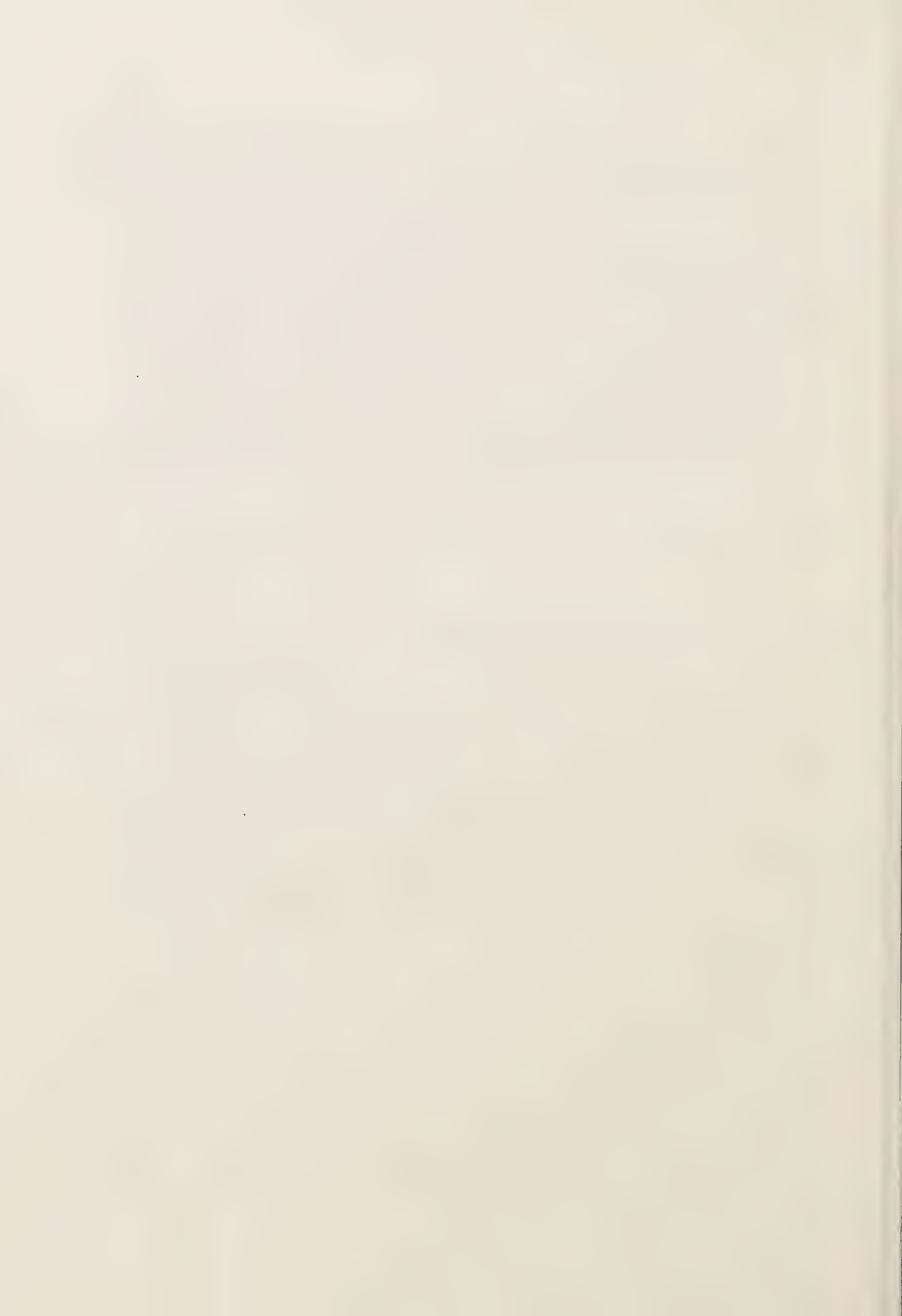
The concentration of active products at the electrode surface will therefore be much lower than if the electrolysis were performed at a stationary or plane electrode. This may be a factor in explaining why no electrode fouling was observed even after very long repeated oxidation.

The accuracy of the coulometric measurements is rather astonishing when the procedure is considered. Diffusion of reactant will normally take place through the filters and during a long run an appreciable loss can occur [13]. The cofactor could diffuse into the intermediate chamber and the counter electrode compartment. The intermediate chamber was occasionally analyzed spectrophotometrically and in all cases the amounts of NAD^+ and NADH were negligible. Both NAD^+ and NADH have a negative overall charge and therefore the migration counteracts diffusion. This effect should be small, however, as the electrolyte concentration is fairly high. Possibly the clay filters are charged so that exclusion takes place and the charge is transported by positive ions.

Financial support from the Swedish Natural Research Council is gratefully acknowledged.

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ADSORPTION AND ELECTROCHEMICAL OXIDATION BEHAVIOUR OF NADH AT A CLEAN PLATINUM ELECTRODE

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(Received 2nd October 1979; in revised form 26th November 1979)

ABSTRACT

NADH can be reproducibly oxidized at a platinum electrode which has been electrochemically cleaned in a buffer without a cofactor. NAD^+ adsorbs slowly and passivates the oxidation of NADH. If the electrode attains a negative potential in NAD^+ or NADH containing solutions, it is passivated. Model experiments were made with AMP and NMN⁺. Linear sweep voltammetry was used to investigate the reaction mechanism. An ECE mechanism is proposed as the main reaction with dimerization of the $\text{NAD}^\cdot$ radical or a disproportionation as alternative routes to NAD^+ .

INTRODUCTION

The electrochemical reduction of the cofactor NAD^+ (nicotinamideadenine-dinucleotide) is very complex. It has been studied extensively, but is still only partially understood. Previous work has been critically reviewed [1,2]. As yet, the oxidation of NADH has been little studied. Early experiments by Burnett and Underwood [3] showed a quantitative recovery of NAD^+ upon electrolytic oxidation of NADH. Blaedel and Jenkins [4,5] determined NADH directly by steady-state voltammetry. Aizawa et al. [6,7] and Johansson et al. [8] made quantitative oxidations and showed that the product was practically 100% enzymatically active. Moiroux and Elving [9,10] studied adsorption effect of NAD^+ on carbon electrode and concluded that in order to control the anodic behaviour of NADH it is essential to control experimental parameters such as electrode material, pretreatment and conditioning of the electrode.

A few mechanistic studies have also appeared. Blaedel and Haas [11] examined the oxidation of some NADH models in acetonitrile. Their results clearly showed that the oxidation proceeded by two stepwise one-electron reactions with formation of an intermediate radical. Leduc and Thevenot [12,13] also studies some model compounds as well as NADH in aqueous solution over a wide pH range and concluded, on the basis of the pH independence of the oxidation wave, that no acid–base reactions were involved. On these ground they also proposed two stepwise charge transfers with the formation of an intermediate radical. Elving et al. [14] studied the oxidation of NMNH (nicotinamidemononucleotide) and NADH in aqueous and DMSO solutions. There was no influence of added bases, in contrast to the findings by Blaedel

and Haas [11], neither did they find any evidence of the mechanism proposed by Leduc and Thevenot [12,13]. They interpreted the results as a direct two-electron reaction. However, in a later review [2] they mentioned the possibility of radical formation, but added that its oxidation is so rapid that the radical intermediate could not be detected. In a recent work by Jensen and Elving [15] it is emphasized that there is still considerable uncertainty about the detailed nature of the electrochemical oxidation of NADH.

In several of the investigations of NADH oxidation, passivation or fouling has been observed, especially in aqueous solutions. In some cases the investigation has been restricted to very low concentrations to reduce these effects. Furthermore the current-voltage curves are irreproducible and dependent on the previous electrode history. Therefore, the first step in this investigation was a time-consuming search for a suitable electrode pretreatment procedure.

A theoretical basis, including diagnostic criteria, for cyclic and linear sweep voltammetry has been developed during the last two decades mainly by Nicholson, Shain et al. [16–21] and by Savéant et al. [22–26].

EXPERIMENTAL

A rotating platinum disc electrode, Tacussel type EDI, with an area of 3.1 mm² was inserted into a Metrohm titration cell, EA 875-5. A double-junction reference electrode, Radiometer K-701, was provided with a Luggin capillary which was positioned within 1 mm of the Pt disc electrode [27]. The outer chamber and the capillary were filled with a buffer solution of the same composition as that in the titration cell. The auxiliary electrode was a Pt gauze inserted into a tube which fitted into the top of the titration vessel, being separated from the test solution by a clay filter.

A precision triangle-wave generator provided the control voltage to a fast potentiostat, which had a hold function and means for setting the initial and final potentials. The current-voltage curves were recorded by an X-Y recorder up to 800 mV s⁻¹, and or a Tektronix 564 storage oscilloscope for faster scans.

A buffer solution containing 0.10 M Na₂SO and 0.005 M Na₄P₂O₇ was adjusted to pH 9.0 with H₂SO₄ and another solution containing 0.10 M Na₂SO₄ and 0.005 M Na₂HPO₄ was adjusted to pH 7.0 in the same way. All solutions were prepared from analytical grade reagents and water purified by reverse osmosis, followed by ion exchange, adsorption and filtration (Millipore Q/RO4). Test solutions were made by adding weighed amounts of β-NADH (Sigma Chemical Co., No. N-8129) to aliquots of the buffer solutions. The final concentration was determined spectrophotometrically at 340 nm, $\epsilon = 6220 \text{ l mol}^{-1} \text{ cm}^{-1}$ [28].

The working electrode pretreatment started by metallographic polishing on rotating felt discs. The electrode was mounted perpendicular to the disc. Polishing was made with 1, 0.25 and 0.03 μm alumina (Struers, Copenhagen). The electrode was then cleaned from particles in an ultrasonic bath, and dipped into hot concentrated sulphuric acid for 1 min. After careful washing with water the electrode was inserted into the cell which contained the buffer solution. The potential was scanned with 100 mV s⁻¹ between -850 and +1000 mV vs. SCE until the adsorption peaks of hydrogen at -800 to -850 mV were

clearly visible and reproducible. The appearance of these peaks was taken as a criterium for electrode cleanliness [29,30]. The electrode was then scanned seven times between 300 and 1000 mV in order to obtain a lower background current.

The electrodes were then moved to another titration cell containing the test solution. The liquid left on the electrodes had been removed by suction to prevent dilution of the test solution. The electrode was rotated to remove bubbles and to ensure thorough mixing. The motor was then switched off and the solution was allowed to quiet down for about 30 s. During this time the potentiostat was in stand-by mode with the electrodes unconnected.

The potentiostat was switched to the operating position and after 5 s a test scan from 300 to 1000 mV vs. SCE was started. After this scan the electrodes were transferred back to the buffer containing the titration vessel and cleaned by repeated scans, as described above, before making the next measurement.

Measurements were made at ambient temperature, 25°C with NADH concentrations of 0.50, 0.86, 2.0, 3.1 and 4.0 mM at pH 9.0 in the pyrophosphate buffer and of 1.0, 2.0, 3.0, 3.8 and 4.5 mM at pH 7.0 in the phosphate buffer.

RESULTS AND DISCUSSION

Adsorption

An electrode, cleaned as described in the experimental section, gives an oxidation wave as shown in Fig. 1, curve A. The peak current depends on the sweep rate, pH and concentration, but the peak potential on the sweep rate only. These dependences are discussed below in connection with the oxidation mechanism. The curve also depends on the previous electrode history. Curve B shows the result when a test scan had been performed in a buffered 1 mM NAD⁺ solution after cleaning and before transfer to the NADH-containing test solution. The oxidation peak is more anodic and broader, i.e. it is more irrevers-

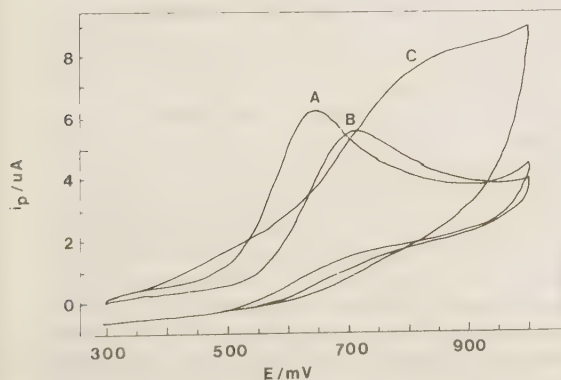


Fig. 1. Oxidation of 1 mM NADH at a stationary Pt electrode with a sweep rate of 50 mV s⁻¹, pH 9.0: (A) at a clean electrode; (B) at a clean electrode after sweeping from 300 to 1000 mV in buffered 1 mM NAD⁺ solutions; (C) at a clean electrode after sweeping from 300 to -850 mV and back in 1 mM NADH.

ible. Curve C shows the oxidation peak obtained when the electrode, after cleaning, had been pretreated by sweeping the potential in a 1 mM NADH solution from 300 to -850 mV and then back to 1000 mV. The Figure shows only the recording between 300 and 1000 mV. At negative potentials the curve was indistinguishable from the background current in a buffer solution. It can be seen that the scanning treatment makes the oxidation still more irreversible.

Moiroux and Elving [10] found that NAD^+ was strongly adsorbed at carbon electrodes. Obviously, it is also adsorbed at the platinum electrode and the effect shows up as a partial blocking of the oxidation of NADH. As there are no prepeaks at sweep rates up to 100 V s^{-1} the adsorption is weaker than on carbon electrodes. If successive sweeps are made in the same solutions starting with a clean electrode but with no cleaning between the sweeps, the first peak is as shown by curve A, the following peaks being successively displaced towards more positive potentials. For fast sweeps the displacement is so small that it is barely noticeable, for slower sweeps — less than 50 mV s^{-1} — the effect becomes more pronounced. This shows that the adsorption of the NAD^+ formed during the oxidation is slow. A comparison with Morieux and Elving [10] shows further that the overvoltage for oxidation is larger on platinum than on carbon. This has also been noted earlier [4]. The blocking after a negative sweep, curve C, is surprising. The effect becomes the same whether a NADH or a NAD^+ solutions is used. It starts in the range of the reduction of the chemisorbed oxygen layer, 0 to -300 mV, depending on pH.

If the electrode is pretreated with HCl instead of H_2SO_4 the peaks becomes more irreversible. If chloride ions are present in the solutions, even in a very low concentration, the peak moves slowly during a few hours towards a more positive position. As platinum forms strong chloro complexes this effect may be specific for the electrode material.

Adsorption of model compounds

Solutions of 1 mM NMN⁺ (nicotinamidemononucleotide) were investigated in the same way as described above for NAD^+ . No adsorption was observed. Oxidation curves for NADH following pretreatment, by voltammetric scanning in NMN⁺, were identical to that of a freshly cleaned electrode (see curve A, Fig. 1). In fact, a passivated electrode could be cleaned by treatment with NMN⁺ present in the buffer solution.

When the experiments were repeated with 1 mM solutions of AMP, (adenosine monophosphate) the results were strikingly different. After a negative scan from 300 to -850 mV and back again, the following oxidation peak of NADH was identical to curve C in Fig. 1. The voltammetric curves in AMP solutions were identical to curves obtained in buffer solutions, i.e. there were no detectable Faradaic reactions. On the other hand, a test scan from 300 to 1000 mV in a buffered solution of AMP had no adverse effect on the NADH oxidation. A difference in behaviour by the two model compounds was thus detected. AMP passivated the oxidation of NADH if a negative scan had been performed in the same way as was found by both NAD^+ and NADH. NMN⁺, however, did not passivate the electrode in the entire potential range used.

The adenine part of the NAD^+ or NADH molecule seems therefore to be

responsible for the passivating effect of a scan to negative voltage (curve C). The weaker passivating effect obtained by a test scan in a NAD^+ solution is not produced by either NMN^+ or AMP, although its parts should be good models for the two nucleotide parts of NAD^+ . Obviously the effect is produced only by the two ring systems in combination. This seems puzzling as a recent study [31] has shown that the interactions between the rings are very weak in solution. In the most probable configurations the rings are not very close together.

Oxidation of NADH

The peak current, i_p of the oxidation peak at a cleaned electrode was measured for a number of scan rates, v , and for five NADH concentrations. The peak currents were plotted vs. the square root of the scan rate. The results in the pH 9.0 buffer is shown in Fig. 2. A similar set of measurements were obtained in the pH 7.0 buffer, a straight line was also obtained in this plot for each concentration. An irreversible two-electron heterogeneous reaction should give a linear plot and the lines should pass the origin according to the theory developed by Nicholson and Shain [16]. Figure 2 shows that the data set does not pass this diagnostic test as the lines obtained for higher NADH concentrations give a positive intercept on the current axis. The slope increased with concentration as expected from the theory.

Figure 3 shows another type of plot, this time the data obtained at pH 7.0 have been used. This plot has also been suggested by Nicholson and Shain [16].

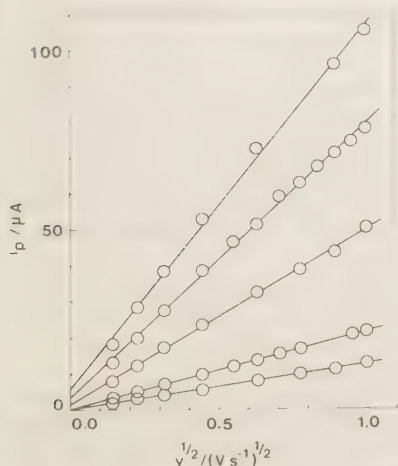


Fig. 2. The peak current on oxidation of NADH at a clean Pt electrode plotted vs. the square root of the scan rate, pH 9.0. The NADH concentrations are, from the bottom upwards, 0.50, 0.86, 2.0, 3.1 and 4.0 mM.

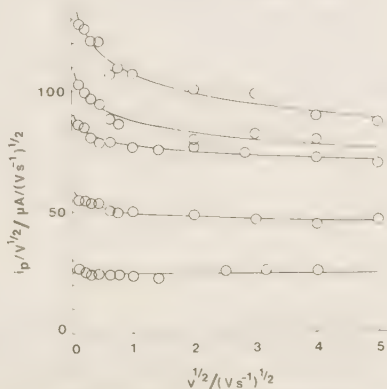


Fig. 3. The peak current divided by the square root of the sweep rate plotted vs. the square root of the sweep rate, pH 7.0. The NADH concentrations are from the bottom upwards, 1.0, 2.0, 3.0, 3.8 and 4.5 mM.

For a direct two-electron transfer [16], for two consecutive one-electron transfer steps [18], as well as for an ECE [17] reaction with a very fast chemical step in between, this plot should give horizontal lines. This is almost the case for the lowest concentration, but it is far from true at the higher. A similar plot was made with the data obtained in the pH 9.0 buffer, the picture is very similar and the conclusions the same. If the $i_p/\sqrt{v}$ is plotted vs. $\log v$ instead of $\sqrt{v}$ as in Fig. 3, straight lines are obtained. The range covered is from 20 to 25 $V s^{-1}$ and the fit to straight lines is very good over the whole range. In fact the curves drawn in Fig. 3 are obtained from regression analysis of the logarithmic plots. The fact that the data yield non-horizontal lines on a logarithmic plot indicates that a chemical reaction of second or higher order is involved [23].

Figure 4 shows a plot of the slopes of the lines obtained when $i_p/\sqrt{v}$ is plotted vs. $\log v$. The slopes are plotted versus the square of the NADH concentration. The points conform to two lines passing close to the origin. This gives evidence for the involvement of a second-order chemical reaction which is pH-dependent.

The previous plots have used only the peak current variation with scan rate. There are also tests which use the peak shape and peak potential [22–26]. Figure 5 shows a plot of the difference between the peak potential and the potential at half the peak current ΔE_p plotted vs. the logarithm of the sweep rate. For a two-electron irreversible reaction the difference should be independent of the scan rate [16]. This is clearly not the case, except at low scan rates. This also gives evidence against a simple two-electron charge transfer. It should also be ob-

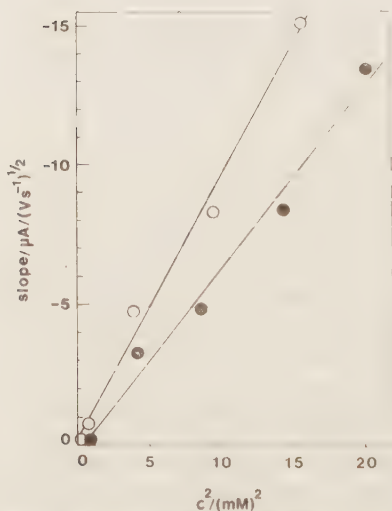


Fig. 4. Diagnostic test for the reaction order, see text for explanation; (○) pH 9.0 (●) pH 7.0.

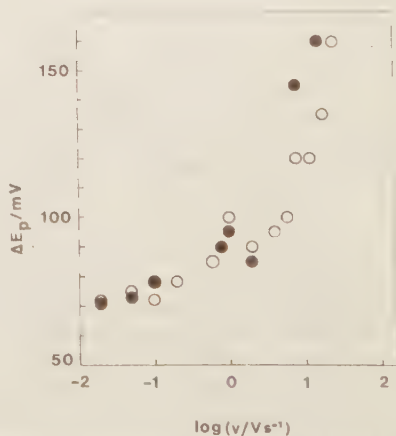


Fig. 5. The potential difference between the peak potential and the potential at half the peak current ΔE_p plotted vs. the logarithm of the sweep rate, pH 9.0. Only two concentrations, 2.0 mM (○) and 4.0 mM (●) NADH, are shown for clarity.

served that the rise occurs at the same scan rate for all concentrations. Figure 5 shows data obtained at pH 9.0, but there is practically no pH dependence as the data for pH 7.0 show coincidence.

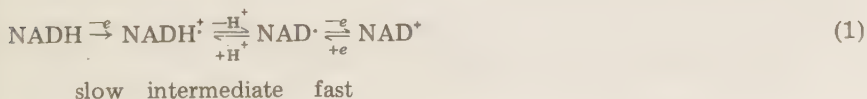
A plot of the peak potential vs. the logarithm of the scan rate yielded a nearly linear relation. Influence of concentration or pH was not found. The lack of features in a plot of this kind gives no evidence for or against a direct two-electron charge transfer [16,17,22–26].

Mechanism

From a previous study [8] it is known that the product from an electrochemical oxidation of NADH is NAD^+ with a yield of at least 99.3%. This NAD^+ is enzymatically active. The experiment mentioned was made with a rotating Pt gauze. Any intermediate products should be mixed with the bulk of solution much more efficiently than at the stationary electrode used in the present study. All reaction schemes must therefore give NAD^+ as the only final product.

As mentioned in the introduction, it has been discussed in the literature whether the reaction is a two-electron charge transfer or involves two single-electron steps. The data shown above clearly show that the direct two-electron alternative is ruled out. The methods used here give possibilities for a more precise diagnostic test than those used earlier [4–15]. Furthermore, as shown by Fig. 3, the effects become much more pronounced at higher concentrations. Previous mechanistic studies using concentrations above 1 mM NADH solutions [14,15], were not able to observe the concentration dependence owing to high background currents.

If one electron is removed from NADH the product will be a radical, $\text{NADH}^\cdot$. If a proton is also removed, another radical $\text{NAD}^\cdot$ will be formed. This radical is known from studies of the reduction of NAD^+ [2], its reduction potential is -1.1 V and it is electrochemically reversible. The possibility of formation of $\text{NAD}^\cdot$ by a combined electron and proton transfer is ruled out as the only initial step. The $\text{NAD}^\cdot$ radical would immediately be oxidized and the overall oxidation would behave as a direct two-electron reaction [18]. The simplest scheme which can account for the facts mentioned above is



This scheme produces a graph of the same shape as Fig. 5 [23], the chemical reaction being of first order. On the other hand, it cannot alone explain the behaviour shown in Fig. 3. From Fig. 4, it was also concluded that a second-order pH-dependent chemical reaction was involved. There are three possibilities to extend reaction (1): firstly $\text{NADH}^\cdot$ could dimerize, secondly $\text{NAD}^\cdot$ could dimerize and thirdly $\text{NADH}^\cdot$ and $\text{NAD}^\cdot$ could disproportionate to NADH and NAD^+ .

The dimer NAD_2 has been studied earlier [2]; it is chemically stable at high pH and it can be quantitatively oxidized to NAD^+ , at about 0 V vs. SCE.

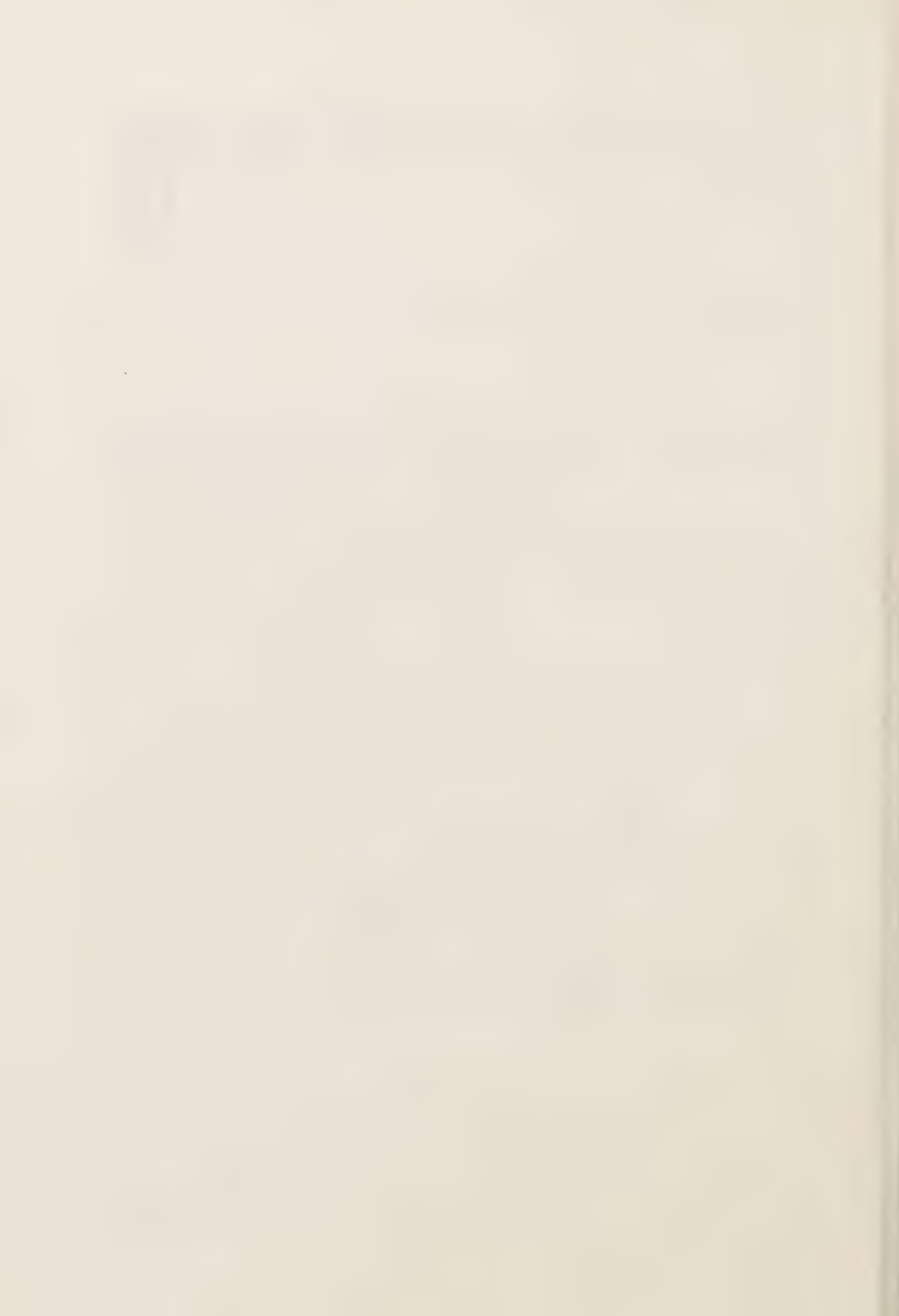
Nothing is known about a dimer NADH[•] and its formation is less likely as it would require dimerization of two charged molecules. Further tests should be performed to give more conclusive information about the two alternatives which are most likely.

ACKNOWLEDGEMENTS

The author thanks Professor G. Johansson for valuable discussions concerning this work and the Swedish Natural Research Council for financial support.

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420 — A STUDY OF THE PRODUCTS FORMED IN THE ELECTROCHEMICAL REDUCTION OF NICOTINAMIDE—ADENINE—DINUCLEOTIDE

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(Revised manuscript received February 25th 1981)

SUMMARY

The reaction products formed when nicotinamide—adenine—dinucleotide (NAD^+) was reduced at a mercury electrode were analysed chromatographically. Reduction at -1.1 V vs. s.c.e. in a one-electron step produces a radical which reacts further into dimers (90% yield). The dimers were separated using reverse phase HPLC and gel-filtration on Sephadex G-15. The wavelength maxima and molar absorptivities of the dimers are given, as well as their NMR-spectra. The dimers were identified and it was found that three stereoisomers of the 4,4'-dimer account for 90% of the dimer mixture and that three 4,6'-dimers are responsible for the remaining 10%. No 6,6'-dimers could be detected. Reduction at -1.8 V produces 1,4-NADH, 50%, 1,6-NADH, 30% and dimers 20%. The yield of the enzymatically active 1,4-NADH increased somewhat with decreasing pH.

INTRODUCTION

Reduction of nicotinamide—adenine—dinucleotide (NAD^+) proceeds in two steps. The first one-electron step at -0.9 V vs. s.c.e. on a mercury electrode produces a radical which can dimerize further. The products are enzymatically inactive. The second step at -1.7 V vs. s.c.e. gives products with some enzymatic activity. These aspects are reviewed in detail by Elving et al. [1]. The complicated nature of the problem of analysing the dimers is emphasized by the fact that at least six different structures are possible. Elving et al. regarded the most likely species to be either a 4,4'- or a 6,6'-dimer. Burnett et al. [2] considered the 4,4'-dimer as the most likely one. They based this conclusion partly on NMR-spectra of the mixture. In some cases model compounds were studied. Biellmann et al. [3] used N-benzyl-nicotinamide and considered all of the species to be the 4,4'-dimer. Carrelli et al. [4] isolated four dimers of 3-cyano-1-methyl-pyridine and identified them as two pairs of stereoisomers of the 4,4'- and the 4,6'-dimer. Stereoisomers are possible because of the asymmetric carbon atoms at the point of dimerization [1]. Thus, considerable uncertainty about which dimer or dimers are actually formed at the reduction of NAD^+ still exists.

The electrochemistry of the second reduction wave of NAD^+ at a mercury electrode, was studied by Elving et al. [5]. In an ordinary carbonate buffer only one reduction wave was seen. Adsorption of NAD^+ increased the reduction potential of the second step into the background discharge. If tetra-ethylammonium chloride (Et_4NCl) was added, NAD^+ was desorbed due to preferential adsorption of the salt. Two well-defined diffusion-controlled waves were seen at sufficiently high Et_4NCl concentrations. Electrolysed solutions contained 54% 1,4-NADH. The dimer content was estimated to 11%. The remaining 35% was thought to be 1,6-NADH, but this was not strictly proven.

In this work an attempt has been made to clarify the uncertainties discussed above. In order to do this, two powerful analytical techniques were used in combination with the electrochemical experiments. Because of differences in size, gel-filtration was used in the separation of dimers from the coenzyme [2]. Separation of NAD^+ and NADH [6] and the dimers was also carried out by reversed phase high-pressure liquid chromatography (HPLC).

EXPERIMENTAL

The electrochemical reduction of NAD^+ (Sigma Chem. Co. N-7004) was made in a three-compartment cell [7] using a three-electrode potentiostat. The cathode was a mercury pool, $\varnothing = 42$ mm, stirred magnetically with a Teflon-coated bar. A saturated calomel electrode was used as a reference electrode. The solution was purged with nitrogen purified with an oxytrap (Altech Cat. No. 4001) which kept the oxygen level below 1 ppm. The working electrode compartment was normally filled with 10 cm^3 buffer containing 1 mM NAD^+ . When the current had decreased to the background level after about $\frac{1}{2}$ h, the solution was removed and cooled to 0°C to prevent decomposition. The reductions were made at -1.1 V and at -1.8 V vs. s.c.e. at 7°C .

Production of reaction products for UV or NMR studies were made with 5 cm^3 15 mM NAD^+ . Four buffers were used: 0.10 M NaHPO_4 , 0.05 M Et_4NCl at pH 7.0; 0.10 M NaHCO_3 , 0.050 M Et_4NCl at pH 8.0; 0.10 M $\text{Na}_2\text{P}_2\text{O}_7$, 0.050 M Et_4NCl at pH 9.0; 0.10 M Na_2CO_3 , 0.050 M Et_4NCl at pH 10.0. The pH adjustments were made with HCl.

All solutions were prepared from analytical grade reagents and water purified by reverse osmosis followed by ion exchange adsorption and filtration (Millipore Q/R04).

Gel-filtration

Gel-filtration on Sephadex G-15 (Pharmacia Fine Chemicals 17-0020-01) was made in a 30 cm long bed, i.d. 1.0 cm, using 0.10 M NH_4HCO_3 , pH 8.2 as an eluent. The flow rate was $0.46 \text{ cm}^3 \text{ cm}^{-2} \text{ h}^{-1}$. The samples, 100 mm^3 (μl), were introduced by a sample loop (Altex rotating valve, Ser. 202). The separated samples were detected with a UV detector at 254 nm (LKB UV-cord 4700).

Gel-filtration using polyacrylamide gels was unsuccessful, probably due to adsorption of the samples to the gel.

Liquid chromatography

The products of the electrolysis were separated by reverse phase HPLC using a programmed gradient (water 30%, ethanol 1–6% and 0.10 M NH_4HCO_3 69–64%). The instrument was a Spectra Physics SP 8000 HPLC chromatograph. The run started isocratically at 1% ethanol and continued with a linear segment of increasing ethanol concentration up to about 4% ethanol. The peaks were eluted during this part of the cycle. Another linear increase in ethanol was linked afterwards to wash the column before the decreasing gradient which restored the initial conditions for the next run.

The column was a C-18 bonded phase (Hypersil, 5 μm , length 200 mm, i.d. 5 mm) operated at 30°C with a flow rate of 1 $\text{cm}^3 \text{min}^{-1}$ resulting in a pressure drop of 1000 psi. The solvents were purged with helium for 15 min before starting the pump. Two UV detectors were used in series (LDC Spectromonitor III and LDC UV monitor 1285) in order to be able to monitor at 254 and 340 nm simultaneously.

Each injection resulted in smaller retention volumes. The column behaviour deteriorated completely after about 20 large samples (500 mm^3 of the 15 mM solution). The column life was much longer when only analytical samples were run. Unidentified reaction products obviously adsorbed on the column. The column could be restored with a rinsing procedure which started with thorough rinsing with water for several hours to remove all traces of the buffer. An ethanol gradient was then used to reach 100% ethanol, which was pumped through the column overnight. A reverse gradient restored the chromatographic system in such a way that precipitation of salts in too strong ethanol solutions was prevented.

In the preparative runs the samples were collected and immediately frozen to -78°C in a bath containing ethanol and solid CO_2 . Collections were made during repeated chromatographic runs to obtain a sufficiently large amount of substance. The collected fractions were freeze-dried for 24 h to a fluffy web-like powder. About 90% of the NH_4HCO_3 was also removed during the drying.

NMR analysis

NMR-spectra were recorded on a Varian XL-100 Fourier transform spectrometer. The chromatographically isolated products were dissolved in 0.5 cm^3 99.95 at. % D heavy water (Ciba-Geigy 57922). The remaining NH_4HCO_3 gave a well-defined pH of 8.2. NMR-spectra were taken at 5 and 28°C. The reference substance used was TMA (tetramethylammonium chloride) $\delta = 3.40$ vs. trimethylsilylpropane sulphonic acid at pH 8.2.

Synthesis of NADH-isomers

Here, 50 mg NAD^+ in 5 cm^3 0.10 M $\text{Na}_2\text{P}_2\text{O}_7$ at pH 9.0 was reduced chemically with a slight excess of NaBH_4 [10]. The reduction produced almost equal amounts of the three isomers 1.2-, 1.4- and 1.6-NADH. They were isolated and freeze-dried as described above and used as reference compounds for the NMR-spectra interpretation.

Unfortunately, the HPLC column used elsewhere in this work had collapsed and an identical one could not separate the 1.4- and 1.2-species completely due to severe tailing of these two isomers. The given NMR-spectra of the 1.2-NADH are therefore taken on the mixture. The molar absorptivities on the other hand are based on data obtained on fractions from the first column and should therefore be correct.

RESULTS AND DISCUSSION

Reduction I

When NAD^+ was reduced at -1.1 V vs. s.c.e. a slightly yellow solution of NAD^+ -dimers was obtained. Its UV data agreed well with those reported previously [1]. The reduction current, I , agreed well with the equation $I = I_0 \times \exp(-\beta t)$ [7] for the 1 mM NAD^+ solution. The residual current was low (10–20 μA) and easily corrected for. The original concentration of NAD^+ (1 mM) had been determined with UV spectrometry ($\epsilon_{260} = 17,800 \text{ l mol}^{-1} \text{ cm}^{-1}$) [8]. From the corrected current–time integral and the known NAD^+ concentration an n -value of 1.00 was obtained.

The products of the electrolysis were separated with gel-filtration as shown in Fig. 1. Alcoholdehydrogenase (ADH) adenosine and adenine were added as

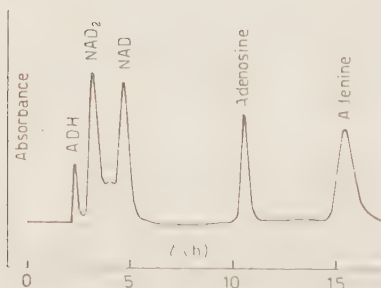


Fig. 1. Gel filtration of a partly reduced 1 mM NAD^+ solution at -1.1 V vs. s.c.e. gives two peaks: The dimeric products NAD_2 are eluted before the smaller NAD^+ molecules. ADH ($M = 80,000$ D), adenosine ($M = 267$ D) and adenine ($M = 135$ D) were added as markers.

markers. At the end of the electrolysis the NAD^+ peak had disappeared completely and there was a corresponding increase in the dimer peak. In the absence of markers there were no peaks at the positions of the adenine or adenosine. A molecular weight determination, to ensure that the second peak consisted of dimers, was not feasible as the products eluted too close to the exclusion limit of the column.

The dimers could be reoxidized to NAD^+ at -100 mV vs. s.c.e. The rate of reoxidation was governed by the electron-transfer kinetics, i.e. the stirring had little effect on the rate, and the current-time relation was far from exponential.

If the integral of the first reduction at -1.1 V was 1.00 the integral on a succeeding oxidation at -100 mV became 0.90. A further reduction gave 0.90 and on the next oxidation became 0.81. The conclusion is that the dimers can account for 90% of the reduction products. The remaining 10% cannot be seen in Fig. 1 and they cannot be reoxidized at -100 mV vs. s.c.e. The adsorption on the HPLC column and the resulting column degradation is probably also caused by these reduction products.

The separated dimers were reoxidized at -100 mV and tested for enzymatic activity with ADH and ethanol. The test indicated that 100% of the NAD^+ was enzymatically active.

The products of the electrolysis were also analysed with a high-resolution HPLC technique. The electrolysis was run to completion so that the sample should contain only the dimers and the unknown reduction products mentioned above. Six different dimer peaks, designated 1 to 6 after the elution order in Fig. 2, were observed. If NAD^+ had been present it should have

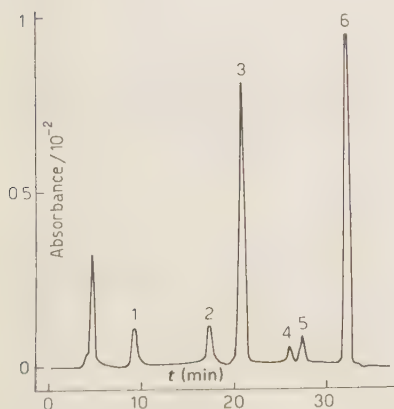


Fig. 2. The products formed on electrolysis of NAD^+ at the first reduction wave on mercury gives a set of dimeric peaks, 1–6, when separated with reversed phase HPLC.

TABLE 1

Relative abundance of the six dimers formed at the reduction of NAD^+ at -1.1 V vs. s.c.e. No pH-dependence was seen between pH 7 and 10

Dimeric species	Relative abundance (%) Corrected for different absorptivity at 260 nm
1	7.4
2	4.3
3	39.6
4	2.7
5	3.2
6	42.8

appeared between peak 1 and 2. The peak near the front is present in the NAD^+ solution from the beginning and is thus not a product of the electrochemical treatment. It has been noted previously [9] that these products are present to a varying degree in the products of different manufacturers.

The relative abundance of each dimer showed little variation with pH, see Table 1. Species 3 and 6 account for more than 80% of this abundance.

The number of chromatographic peaks first suggests that they represent the six possible combinations of the $\text{NAD}^{\cdot-}$ -radical, namely the 2.2'-, 2.4'-, 2.6'-, 4.4'-, 4.6'- and 6.6'-dimers. As the carbon-carbon bonds formed are asymmetrical the total number of stereoisomers becomes 21, and therefore Fig. 2 may show any six of the 21 stereoisomers [1].

Identification and investigation of the dimers

UV-spectra

The UV-spectrum was recorded for a batch of each dimer fraction shown in Fig. 2. Each solution was then oxidized at -100 mV and measured again in the

TABLE 2

Absorptivities and absorption maxima for the six dimeric species

Dimeric species	$\epsilon_{260}/\text{l mol}^{-1} \text{ cm}^{-1}$	ϵ nicotinamide/ $\text{l mole}^{-1} \text{ cm}^{-1}$	λ max nicotinamide/nm
1	31300	7800	342
2	37600	7300	348
3	32900	7100	335
4	38100	6500	354
5	37000	7200	345
6	30200	7400	343

spectrophotometer. As it is known that the latter oxidation quantitatively produces NAD^+ whose molar absorptivity is also known, the data can be used to calculate the original dimer concentration in each fraction. The molar absorpti-

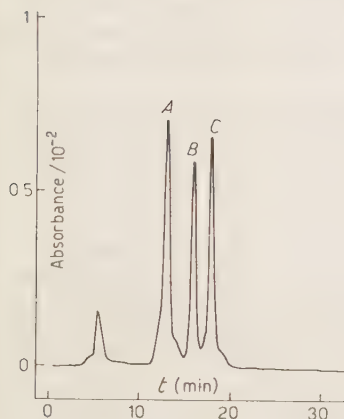


Fig. 3. As NAD^+ is reduced in solution with NaBH_4 almost equal amounts of three NADH isomers are produced: A = 1,6-NADH; B = 1,4-NADH; C = 1,2-NADH. The three isomers are easily separated with reverse phase HPLC.

vities of the dimers can consequently also be calculated, see Table 2. The table also shows the wavelengths at the maxima resulting from the nicotinamide part of the molecule. The given molar absorptivities are those at the wavelength maxima.

It was necessary to purge the solutions with nitrogen before dispensing them into the coulometric cell or there would be oxidation by dissolved oxygen. The mercury acted as a catalyst so that if oxygen was freely supplied the oxidation would be complete within 1 h. The product was the same as in the electrochemical oxidation, i.e. NAD^+ .

The mixture of monomeric NADH-isomers produced with NaBH_4 was also

TABLE 3

Absorptivities and absorption maxima for the three NADH-isomers

Species	$\epsilon_{260}/\text{l mol}^{-1} \text{ cm}^{-1}$	$\epsilon \text{ nicotinamide}/\text{l mol}^{-1} \text{ cm}^{-1}$	$\lambda \text{ max nicotinamide}/\text{nm}$
1,2-NADH	15700	6000	395
1,4-NADH	14400	6200	340
1,6-NADH	21300	6500	345

separated chromatographically, see Fig. 3. The separated products were each investigated in the UV spectrophotometer and then oxidized back to NAD^+ , using a rotating platinum gauze electrode held at +700 mV vs. s.c.e. [7]. The oxidation was quantitative and therefore a new spectrophotometric measurement could be used for evaluation of the original concentration of the isomers. In each case the enzymatic activity of the product, NAD^+ , was according to expectations.

Table 3 shows the spectrophotometric data calculated from this experiment. The molar absorptivity of 1.4-NADH which now is based on that of NAD^+ [8] is consistent with the value used as standard for the data in Table 2. The wavelength maxima given in Table 3 are identical to those found from spectrophotometric measurements on the mixture [10].

The major part of the absorbance at 260 nm is due to the adenine ring, but there is also a contribution from the reduced nicotinamide part of the molecule. As can be seen the contribution is especially strong for 1.6-NADH. For model compounds such as NMNH it is known that the 1.6-dihydro species has a greater absorptivity at 260 nm than both the 1.2- and the 1.4-NMNH [11].

None of the dimers of Table 2 shows absorbance maxima around 395 nm which, according to the data given in Table 3, should have been the case if any 2.x'-dimer had been present. This was also concluded by Elving et al. [1] from a spectrophotometric analysis of the mixture.

Table 2 shows that there is one group of dimers having a mean molar absorptivity of $31,500 \text{ l mol}^{-1} \text{ cm}^{-1}$ (1; 3; 6) and another group with a mean value of $37,600 \text{ l mol}^{-1} \text{ cm}^{-1}$ (2; 4; 5). A comparison with the molar absorptivity in Table 3 indicates that the first group should consist of 4.4'-isomers as the 2.x'-dimer was excluded due to the position of the wavelength maxima. The group of dimers with high molar absorptivities at 260 nm must thus be of either the 4.6'- or the 6.6'-type.

Hydrolytic stability

A solution of each dimer was kept at room temperature at pH 8.2 in NH_4HCO_3 buffer. The solutions were collected directly from the HPLC outlet. The UV absorptions were measured daily at the wavelength maxima of the nicotinamide groups and the result is shown in Fig. 4.

The dimers 1, 3 and 6 form a group in this respect as they did in molar absorptivity. The 2, 4 and 5 dimers are less stable and they form a group distinctly different from the first.

The good stability is remarkable compared to that reported for the unseparated mixture [5]. The different buffers — phosphate and NH_4HCO_3 — should not give these quite large differences. Only in the presence of a catalyst and oxygen was the stability of the separated dimers as low as that given [5] for the mixture. The stability of NADH isomers in alkaline solutions has been previously reported by Chaykin et al. [13].

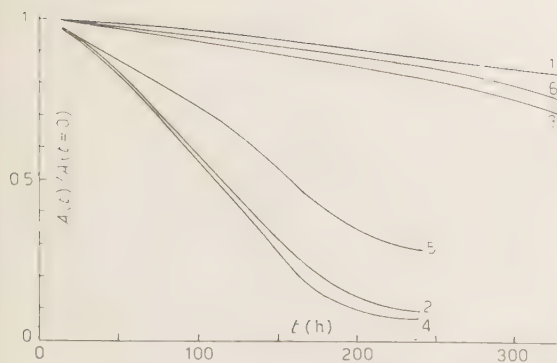


Fig. 4. A stability test of the six dimers run over almost two weeks in $0.10\text{ M NH}_4\text{HCO}_3$, pH 8.2, shows a greater stability for dimers 1, 3 and 6 than for dimers 2, 4 and 5. The measurements of the absorbance were made at the absorption maxima originating from the nicotinamide rings, see Table 2.

NMR-spectra

The NMR-spectra of the six dimers are shown in Fig. 5. It is clear that the spectra of species 1, 3 and 6 are similar to that of 1.4-NADH [12]. The proton resonances AC(2)H and AC(8)H in the adenine moiety are seen at 5.0 and 5.2 ppm vs. TMA (tetramethylammonium chloride). The spectra of dimers 2, 4 and 5 are also similar to the other three, but differ in one important respect. The single signal corresponding to two proton resonances at 3.8 to 4.1 ppm for the 1, 3 and 6 dimers are split into two separate signals corresponding to one proton each. These signals and those between 0 and -1 ppm are crucial in the identification of the dimers. They also show that the nicotinamide ring exists in a reduced state. The oxidized ring has an aromatic structure and the corresponding proton resonances are found between 5 and 6.5 ppm [12].

In order to assign the resonances of the acquired spectra, 1.2- and 1.6-NADH were also prepared and studied (Fig. 6). The NADH-isomers should be good model compounds for obtaining the chemical shifts for the proton resonances in the reduced nicotinamide rings. Using the assignments for 1.4-NADH by Sarma et al. [12] and for the methyl- 1.2- and 1.6-dihydropyridine isomers by Schmidt et al. [14], it is possible to correlate the different signals to the appropriate proton resonances. Table 4 shows the frequencies of the assigned protons in the three nicotinamide rings. Although Schmidt et al. used CDCl_3 instead of D_2O as solvent, it should be possible to compare the chemical shifts and coupling constants. No large solute/solvent interactions are expected between the nicotinamide ring protons and these solvents. In fact all chemical shifts agree within 0.4 ppm, except for the NC(5)H of 1.2-NADH which is found at an unexpectedly low field (Fig. 6). However, the signal pattern at 4.40 ppm (two

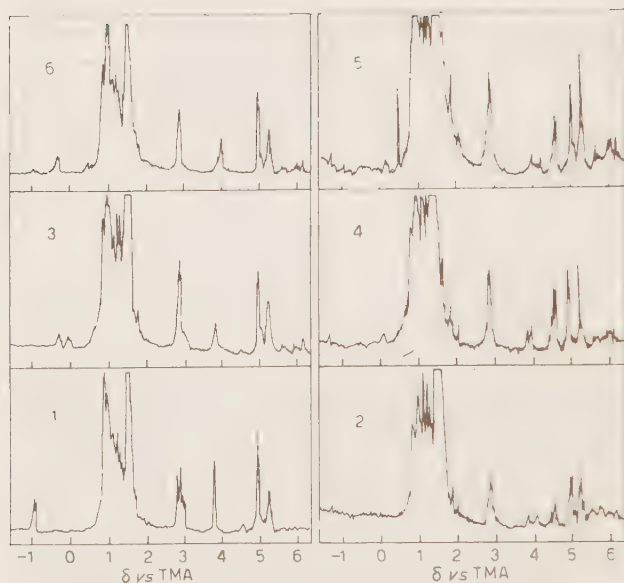


Fig. 5. The NMR-spectra of dimers 1–6 were run on a 100 mHz Varian instrument in 99.95% D_2O . TMA (tetramethylammonium chloride) was used as a reference. The temperature was $28^\circ C$ and the pH 8.2. The pulse length was 1.5 s and the number of pulses for each sample were (1) 4000; (3) 2500; (6) 3100; (2) 4200; (4) 7000; (5) 11,000. The small peaks at 6 ppm and just below originate from decomposed NAD_2 . The unresolved peaks between 1.7 and 0.8 arise from HDO present and the ribose protons.

slightly overlapping doublets) agrees well with that expected for the NC(5)H resonance of 1.2-NADH.

The coupling constant $J_{5,6} \approx 5$ Hz reappears in the signal at 3.69/3.64 ppm of the NC(6)H resonance. The signal at 2.75/2.83 ppm cannot definitely be ascribed to the NC(4)H as the NC(6)H of 1.4-NADH (Fig. 6) also appears at this chemical shift. The coupling constant of the model compound given by Schmidt et al. [14] was $J_{4,5} = J_{5,6} = 6.5$ Hz. The two protons in the 2-position are not seen and the signal is probably hidden under the unresolved ribose resonances. The most important signal of this spectrum is the chemical shift of NC(6)H which appears at a higher field than the NC(2)H resonance of 1.4-NADH.

In the spectrum of 1.6-NADH (Fig. 6) all the chemical shifts in the nicotinamide ring agree quite well with the model compound studied by Schmidt et al. [14]. The NC(2)H resonance is found at 3.93 ppm, 0.3 ppm lower than the NC(6)H signal in the 1.2-isomer. The NC(4)H resonance appears at 2.65 ppm

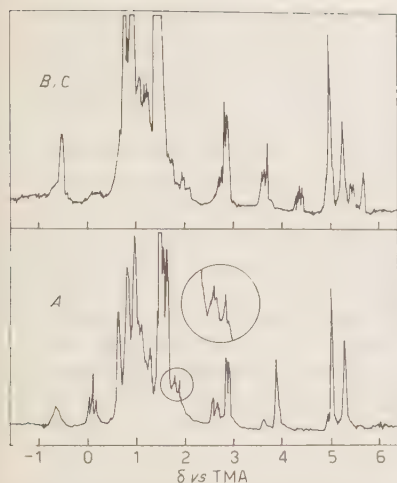


Fig. 6. The NMR-spectra of the three isomers A = 1.6-NADH, B = 1.4-NADH and C = 1.2-NADH were run on a 100 MHz Varian instrument in 99.95% D₂O. TMA was used as a reference. The temperature was 28° C and the pH 8.2. The isomers B and C were not separated and therefore both species appear together. The information obtained was still sufficient as the 1.4-NADH isomer spectra were simple to run separately as they may be bought in batches. The enlarged segment shows the NC(5)H resonance at 1.83/1.93 ppm for 1.6-NADH.

with a coupling constant $J_{4,5} \cong 10$ Hz. The same coupling constant reappears in the NC(5)H resonance, giving rise to a doublet of triplets at 1.93/1.83 ppm on the slope of the HDO-peak. The coupling constant $J_{5,6} \cong 2$ Hz in the weak but clear triplets is also found in the NC(6)H doublet resonance at 0.69/0.67 ppm. The coupling constants of the model compounds were $J_{4,5} = 10$ Hz and $J_{5,6} = 3.8$ Hz [14].

In the spectrum of 1.6-NADH (Fig. 6A) the signal at -0.6 ppm is an unknown

TABLE 4

Protons assignments in the nicotinamide ring ppm v.s. TMA. The pH was 8.2 and the temperature 5 (parenthetical) and 28° C

Species	NC(2)H	NC(4)H	NC(5)H	NC(6)H
1,2-NADH	—	2.75/2.83	4.45/4.37/4.41/4.32	3.69/3.64
1,4-NADH	3.73 (3.72)	-0.45 (-0.54)	—	2.83/2.75 (2.80/2.72)
1,6-NADH	3.93	2.71/2.61	1.83/1.93	0.69/0.67

contaminant from the buffer solution. In fact, it is also visibly present in Fig. 6B, C just under the NC(4)H resonance of 1.4-NADH. The triplet at 0.15 ppm is also an unknown contaminant not present in a spectrum of an unseparated mixture of the isomers after reducing NAD⁺, as described in the experimental section with NaBH₄.

Using the information obtained about the chemical shifts for the different protons in the NADH-isomers, it is possible to make a reliable interpretation of the dimer spectra. Only one signal is seen at about 3.9 ppm for the three 1,3 and 6 dimers embodying two identical proton resonances. This implies a symmetric dimer such as 2.2', 4.4' or 6.6'. The 2.2'-dimer is ruled out as no signals are present in the vicinity of 4.40 ppm, where the NC(5)H resonance would have been found. The 2.2'-dimer is also ruled out by the signals with negative chemical shifts vs. TMA. These signals also exclude the 6.6'-dimer, since they occur at too high a field for any NC(6)H resonances. On the other hand, the 4.4'-dimer should have the NC(4.4')H resonance in this region.

The conclusion is therefore that these dimers (1, 3, 6) are 4.4' structural isomers. The most important chemical shifts for the identification are shown in Table 5.

The spectra of dimers 2, 4 and 5 show at least one significant difference compared to the spectra of 1, 3 and 6. The single peak at 3.8 ppm is split into two (Fig. 5). The appearance of the split peaks manifests an unsymmetrical dimer such as a 2.4' or 4.6' species. The low field at which these signals appear makes a 2.4'- or a 2.6'-dimer highly unlikely. The NC(2)H resonance of a 2.x'-ring would probably not shift from 3.65 ppm to 3.9–4.2 ppm in the 2, 4 and 5 dimers. However, the chemical shifts of the NC(2.2')H signals of a 4.6'-dimer would be expected in this region. That a 4.x'-dimer is present is also shown by the presence of the signals at -0.1 ppm embodying one proton resonance each.

Unfortunately, a complex pattern of signals at 4.59–4.54 ppm makes doubt-

TABLE 5

Proton assignments in the nicotinamide rings ppm vs. TMA. The pH was 8.2 and the temperatures 5 (parenthetical) and 28°C

Dimeric species	NC(2,2')H	NC(4,4')H	NC(6,6')H
1	3.83 (3.81)	-0.90/-0.96 (-1.16)	2.86/2.81 (2.84)
3	3.80 (3.78)	-0.05/-0.30 (-0.03/-0.43)	Not resolved from the AC(1')H
6	3.96 (3.94)	-0.31/-0.35 (-0.38/-0.42)	Not resolved from the AC(1')H
2	4.06/3.86	Too few pulses to be seen	Not resolved from the AC(1')H
4	3.96/3.88	-0.12 one proton	Not resolved from the AC(1')H
5	4.16/3.95	-0.14 one proton	

ful the exclusion of the 2. α '-dimer on the grounds of a lack of NC(5)H resonance at 4.40 ppm (Fig. 5). The peaks due to this proton could be hidden under the complex resonances mentioned above. The obscuring peaks stem from a low concentration of degradation products from the C-18 phase in the HPLC column. They are seen clearly in these spectra because of the larger number of pulses needed for the more dilute samples of dimers 2, 4 and 5. However, the low field at which the split peaks of 2, 4 and 5 appear should be sufficient to prove conclusively the presence of a 4.6'-dimer.

The combined results of the UV- and NMR-spectral analysis, therefore, show that the six chromatographic peaks consist of two different sets of structural isomers.

It was mentioned above that the reduced carbons at the site of dimerization are asymmetric centers. Therefore, there are actually four possible 4.4'-dimers. If the two asymmetric positions are called A and B (Fig. 7), then the four possible dimers may be labeled as (4A-4A), (4A-4B), (4B-4A) and (4B-4B) dimers. Here (4A-4B) and (4B-4A) are equivalent configurations and (4A-4A) and (4B-4B) are mirror images in the nicotinamide parts [15]. In view of the relative amounts of dimers produced (Table 1), dimers 3 and 6 are probably the mirror images and consequently dimer 1 is the (4A-4B) species. However, in the case of the 4.6'-dimer the (4A-6B) and (4B-6A) species are not only identical but also mirror images. Only three 4.6' chromatographic peaks are visible instead of a possible four. It is reasonable to assume that the (4A-6B) and (4B-6A) species would be more difficult to separate with HPLC than the (4A-6A) and the (4B-6B) dimers. Thus, the chromatographic peak 2 would consist of the (4A-6B)/(4B-6A) pair. Peaks 4 and 5 would then be the (4A-6A) and (4B-6B) species.

Reduction II

When the reduction of NAD^+ was carried out at -1.8 V vs. s.c.e. a slightly yellow solution was obtained. The high background current (100 – 200 μA) prevented an accurate coulometric analysis. Figure 8 shows the HPLC chromatogram of the reaction mixture after completion of the reduction. It can be

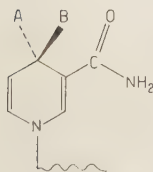


Fig. 7. The asymmetry of a reduced site in the nicotinamide ring is illustrated by the two unequivalent positions A and B.

seen that the same dimers appear as in Fig. 2, although in different relative amounts. The ratio $\Sigma(1, 3, 6)/\Sigma\text{I}^\ominus$ -dimers decreased from 90% when reducing at -1.1 V to below 80% at -1.8 V (Table 6). There are also two new peaks A and

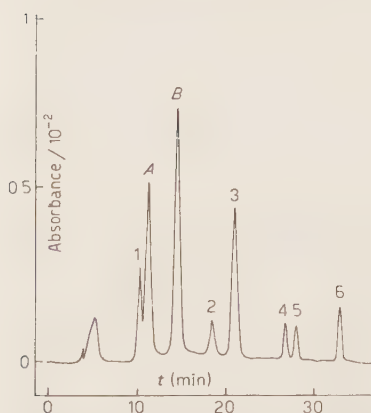


Fig. 8. The products formed on electrolysis of NAD^+ at the second reduction wave gives a set of dimeric peaks 1–6 and two new peaks A and B, when separated with reversed phase HPLC. The products A and B were identified as 1,6- and 1,4-NADH.

TABLE 6

Relative composition (%) of the products formed when reducing NAD^+ at -1.8 V vs. SCE at four different pH. The relative area was corrected for the different absorptivities at 260 nm

Species	pH			
	7	8	9	10
1,6	15.7	20.7	25.9	31.1
1,4	63.3	56.8	57.4	50.1
1	7.8	6.3	5.5	4.5
2	1.5	2.5	1.4	1.8
3	7.1	5.2	6.1	7.7
4	1.3	2.2	1.0	1.2
5	1.1	2.2	1.1	1.5
6	2.2	4.1	1.6	2.1
Σ NADH isomer	79	77.5	83.3	81.2
Σ NAD_2	21	22.5	16.7	18.8
$\Sigma(1, 3, 6)/\Sigma\text{I}^\ominus$ -dimers	81	69	79	76

B. A combined separation using first gel-filtration and then HPLC shows that A and B are of the same molecular size as 1.4-NADH. The analysis also confirmed that the molecular weight of 1-6 corresponded to that of dimers. UV spectroscopy and comparison with retention data from standards showed that peaks A and B consisted of 1.6-NADH and 1.4-NADH respectively. If any 1.2-NADH had been present it should have appeared between peaks B and 2 in Fig. 8.

The enzymatic activity of peak B was verified, as well as the absence of enzymatic activity of peak A. The test was made using CH_3CHO and ADH.

Table 6 shows the relative abundance of each species at different pH. The relative amount of enzymatically active NADH increased slowly with decreasing pH from 50% at pH 10.0 to 63% at pH 7.0. At the same time the relative amount of 1.6-NADH decreased linearly from 30 to 15%. The quantity of dimers was practically constant at about 20%.

The dimer composition is, however, slightly different at this voltage than that at -1.1 V. The dimer composition at -1.1 V was almost independent of concentration, as found when 1 and 15 mM NAD^+ solutions were reduced.

CONCLUSIONS

Reduction of NAD^+ at -1.1 V vs. s.c.e. produces a dimer mixture (90%) and some products (10%) which could not be oxidized at -100 mV. The dimers were identified as two sets of stereoisomers of 4.4'- and 4.6'-dimers. The relative abundance of dimers was independent of pH and concentration. Almost 90% of the dimers consist of the three 4.4' stereoisomers and 10% of the three 4.6' stereoisomers. No 6.6'-dimer-isomers could be detected.

Reduction of NAD^+ at -1.8 V produces mostly 1.4-NADH (50–63%) and 1.6-NADH (15–30%), as well as dimers (20%). As an accurate coulometric evaluation was impossible it is not known whether products which escaped chromatographic detection were also formed. It is remarkable that no 1.2-NADH is formed, as about equal amounts of the three isomers are formed in the reduction with NaBH_4 .

The results presented confirm most of the conclusions drawn by Elving and co-workers from analysis of mixtures [1], except the fact that the probability of forming 6.6'-dimers is much smaller than expected from sterical considerations.

ACKNOWLEDGEMENTS

The author thanks Professor G. Johansson and Professor J. Sandström for valuable discussions concerning this work, and Mr. I. Csiky who assisted in the chromatographic analysis. The author also acknowledges the help of Dr. T. Drakenberg who made the NMR analysis possible.

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Catalytic Oxidation of Reduced Nicotinamide Adenine Dinucleotide by Graphite Electrodes Modified with Adsorbed Aromatics Containing Catechol Functionalities

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4-[2-(2-Naphthyl)vinyl]catechol (NSCH₂) and 4-[2-(9,10-ethanoanthracen-9-yl)vinyl]catechol (ASCH₂) were adsorbed on graphite electrodes. The naphthalene and the ethanoanthracene ring systems were used as anchors to the graphite. The catechol group is free to move out into the electrolyte solution. The electron transfer between the 1,2-hydroquinone functionality and the graphite was fast. The surface coverage was at most 9×10^{-9} mol cm⁻². The coenzyme reduced nicotinamide adenine dinucleotide (NADH) could be catalytically oxidized by the immobilized mediating groups. The overvoltage of the NADH oxidation decreased from 410 mV vs. SCE at the unmodified graphite electrode to 185 mV at the NSCH₂ covered electrode at pH 7.0. The reaction rate of the ASCH₂ covered electrode was lower than that of the NSCH₂ electrode. After 30 min of continuous electrochemical cycling of pH 7.0, 30% of the original coverage remained for the NSCH₂ electrode.

The coenzyme reduced nicotinamide adenine dinucleotide (NADH) can only be oxidized electrochemically at high overvoltages (1). The overvoltage at pH 7 is about 1.1 V at carbon (2) and 1.3 V at platinum electrodes (3). If the electrode has been pretreated and if the NADH concentration is at most 0.1 mM, the product at platinum electrodes is enzymatically active NAD⁺ (4). Electrodes modified so that NADH could be oxidized more easily should open up a new field of applications both in analysis and in biotechnology.

About 300 hydrogenases are known which are dependent on NADH or NADPH as redox transfer agents.

Oxidation of NADH to enzymatically active NAD⁺ in homogeneous solution can only be made with a few oxidizing agents, often called mediators.

Surface modifications have been tried as a means to reduce the overvoltage of the electrochemical oxidation. The strategy has been to immobilize functional groups with known mediating properties on the surface. Blaedel and Jenkins (5) could reduce the overpotential with 0.2 V to 0.45 V vs. SCE by an electrochemical pretreatment of the electrode. They presumed that hydroxyl, carbonyl, and quinone functionalities were produced. Tse and Kuwana (6) immobilized 1,2-hydroquinones via covalent bonds to the surface of pyrolytic graphite and showed that the functionalities had catalytic activity. The overpotential decreased further about 0.2 V compared to Blaedel and Jenkins. The work demonstrated the feasibility of making catalytic electrodes by the immobilization of mediating functional groups.

Degrand and Miller (7) made a polymer from poly(methacryloyl chloride) and dopamine which adsorbed on the surface of vitreous carbon. The decrease in overvoltage was about 0.25 V. The surface coverage and lifetime were improved compared to the electrode made by Tse and Kuwana.

It has been observed that a number of polycyclic aromatic compounds adsorb strongly to graphite surfaces. Anson and co-workers studied the electrochemistry of 9,10-phenanthrenequinone (8, 9) and 1-(9-phenanthrene)-2-(4-pyridine)ethene (10) adsorbed on graphite. Laviron et al.

(11) reported on the electrochemistry of benzo[c]cinnoline adsorbed on graphite. Coenzymes from the flavine group were studied in our laboratory (12, 13). The flavines adsorbed strongly to graphite, and the electron transfer between the functional group and the graphite was quite rapid.

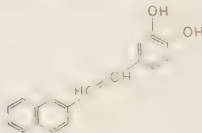
As it is known that some 1,2-quinones can be mediators in homogeneous solution there is a possibility that the adsorbed 1,2-quinones should be able to mediate electron transfer from NADH. No such effect could be seen for the 9,10-phenanthrenequinone (6) or for a greater number of condensed aromatic quinones studied preliminarily in our laboratory. The position of the adsorbed quinone functionality, which probably lies flat along the surface, seems to be unfavorable for electron exchange with NADH. Utilization of this mediator seems to be possible only if the functional group can be raised somewhat from the surface. The success reported by other types of immobilization (6, 7) supports this belief.

This paper reports on the adsorption of two types of compounds which have a 1,2-hydroquinone functionality in a side ring connected to a larger aromatic ring system. The larger aromatic ring systems anchor the compounds to the graphite surface and the smaller catechol acts as a mediator. The anchoring aromatic ring also provides a fast electron exchange between the graphite electrode and the adsorbed functionality.

A voltage facilitated immobilization of phenazine methosulfate mediator on graphite has simultaneously been made in our laboratory (14). These electrodes also showed catalytic activity for the NADH oxidation.

EXPERIMENTAL SECTION

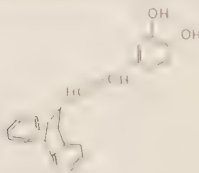
Syntheses. 4-[2-(2-Naphthyl)vinyl]catechol (I) was synthesized



from 2-naphthalene aldehyde and 3,4-dimethoxybenzyl alcohol. The dimethoxybenzyl alcohol was treated with PCl_5 in dry ether at 0°C according to Carman and Shaw (15) to produce the acid chloride. The triphenylphosphonium salt was made by boiling the chloride with triphenylphosphine in dry toluene at 110°C for 48 h (16). The formed precipitate was washed with dry cold ether. A Wittig reaction was made between a slight excess of the phosphonium salt and 2-naphthalene aldehyde dissolved in methylene chloride by addition of 50% aqueous sodium hydroxide. The mixture was agitated vigorously for 20 min (17). The organic layer was separated and the solvent was evaporated.

The product was purified by passing a solution of the product in petroleum ether-ethyl acetate 50:50 through a column of silica gel 60 as described by Still et al. (18). Only a small amount of *cis*-stilbene is formed due to steric hindrance. Furthermore the *cis* and *trans* isomers elute in separate fractions from the column. Only the *trans* form was collected. The ether groups were cleaved by treatment with trimethylsilyl chloride and sodium iodide in dry acetonitrile (19). The necessary reaction time was considerably longer than the one reported (19), probably due to steric hindrance between the ortho silyl ethers formed prior to hydrolysis.

The 4-[2-(9,10-ethanoanthracen-9-yl)vinyl]catechol (II) was



made from anthracene, acrolein, and dimethoxybenzyl alcohol.

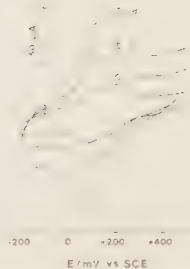


Figure 1. Cyclic voltammogram of NSCH_2 adsorbed on graphite in 0.1 M phosphate buffer, pH 7.0, surface coverage 0.7×10^{-9} mol cm^{-2} , starting potential -100 mV vs. SCE, and sweep rate (a) 200, (b) 100, and (c) 50 mV s^{-1} .

In the first step 9,10-ethanoanthracene carboxaldehyde was synthesized by a Diels-Alder reaction between anthracene and acrolein in toluene during 16 h at 150°C in a steel bomb (20). The reaction mixture was filtered and the solvent of the filtrate was evaporated. The aldehyde was dissolved in methanol and any residual anthracene was removed by filtration. The aldehyde monohydrate was formed by adding water and the precipitate was filtered off. The monohydrate was transformed back to the aldehyde at 100°C under vacuum. The steps of the synthesis and the purification were the same as described above for the compound I. The products, I and II, were identified by IR, NMR, mass spectrometry, and elemental analysis.

Measurements. The electrodes were spectrographic graphite, 6.1 mm o.d. (RW 003 Ringsdorf-Werke, GMBH). They were polished with emery paper (Tufak Durite Waterproof Paper Closecote 600-A Type 1) put on a rotating disk polishing machine. The polishing was performed under a continuous flow of deionized water for 5 min. After that, the electrodes were dried in air. Before mounting the electrodes in the holder for the electrochemical experiment, a rubber tube (6.0 mm i.d., isoversin, Kebo Grave) was slipped over in order to avoid electrical contact between the sides of the electrodes and the solution. This was done from the opposite end of the polished surface in order to avoid affecting the surface mechanically or chemically. The edge of the tube was put just in line with the electrode surface.

The electrochemical surface area was identical with the geometric one when measured in a 2 mM ferrocyanide solution using both a stationary and a rotating disk arrangement. The rubber tubing as well as the sides of the electrodes were quite hydrophobic and no creeping of solution between the electrode and the rubber tubing was detected. Measurements were made with either triangular sweep voltammetry or with a rotating disk assembly (Tacussel EDI) using the SCE as a reference electrode.

All measurements were made in buffers made from 0.1 M pyrophosphate (pH 5.0, 6.0, and 9.0) or 0.1 M phosphate (pH 2.0, 3.0, 4.0, 7.0, 8.0, and 11.0).

The synthesized products were first dissolved in methanol-ethanol 80:20. An equal volume of aqueous 0.1 M hydrochloric acid was added when the dissolution was complete. The resulting solution was about 0.5 mM. The graphite rods were dipped into this solution under slow agitation for 3–10 min depending on the desired coverage.

The purity of the synthesized products was checked by HPLC on $\mu\text{C}18$ -CN, -NH₂, and -C₈, Hypersil ODS-C18 (all 5 μm), and $\mu\text{Porasil-SiOH}$ (10 μm) columns using several different methanol ethanol mixtures as well as acetonitrile as solvents.

RESULTS AND DISCUSSION

Electrochemistry of Adsorbed NSCH_2 . Figure 1 shows cyclic voltammograms of the NSCH_2 covered electrode, obtained in a buffer solution with no soluble NSCH_2 present. The adsorption of NSCH_2 had taken place during 3 min from the solution specified in the Experimental Section. After that the electrode had been washed thoroughly with deionized water. The figure demonstrates the properties of the adsorbed

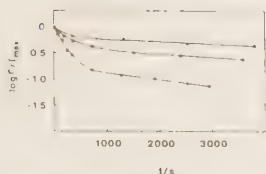


Figure 2. Desorption of NSCH₂ vs. time in an unstirred solution: (▲) pH 5.0; (●) pH 7.0; (■) pH 9.0 and 11.0.

layer. Two peaks are observed. The peak current is linearly proportional to the sweep rate over a range of 25–600 mV s⁻¹ indicating a diffusionless system. The separations between the first anodic and the corresponding cathodic peaks are 40, 50, and 60 mV for the three sweep rates. The corresponding peak separations for the second peaks are 20, 25, and 35 mV. For a fully reversible reaction in which no reactants from the solution take place, the peak separation should be zero (20). The relatively small peak separations indicate fairly fast electrochemical reaction rates (21). If the coverage of adsorbed species is decreased even smaller peak separations are obtained (22). The apparent rate constants, calculated according to these references, were 3–6 s⁻¹, about the same for both steps. The two peaks on both the anodic and cathodic sweeps indicate that the reactions occur in two one-electron steps. The intermediate should be the semiquinone, resonance stabilized by the double bond to the naphthalene ring. A further indication that the reaction proceeds with a one-electron mechanism is that the peak widths at half-height are 90–95 mV (theory 90.6/n mV (23)). A plot of the peak current over the charge vs. the sweep rate also gives evidence of two separate electron steps, as *n* values close to 1 are obtained from the slopes (8). The two electrochemical peaks cannot be caused by two different species as these would have been separated by at least one of the HPLC columns mentioned in the Experimental Section.

The electron transport can take place through different mechanisms. Electrons can be transferred through the double bond resonance structure between the catechol and the anchoring naphthalene ring. The catechol is free to interact directly with the surface so that the electron transfer might also take place through a flip-flop movement. Even an electron tunneling mechanism seems probable.

A plot of *E*_{1/2} vs. pH from pH 1 to 9 gives a slope of 59.4 mV/pH unit at 25.0 °C. It can therefore be concluded that an equal number of electrons and protons is involved in the two electrode reactions. The *E*_{1/2} at pH 7.0 for the two waves were +95 and 190 mV, respectively.

The amount of NSCH₂ on the electrode increased asymptotically with the time spent in the adsorption solution. There was a leveling off after about 60 min. The highest coverage was around 9 × 10⁻⁹ mol cm⁻². The shape of the cyclic voltammograms changes with coverage. The two peaks merge at high coverage and the separation between the anodic and cathodic peaks increases (23).

Stability. Figure 2 shows the rate of desorption of NSCH₂ from the graphite at four different pH values. The measurements were made in unstirred solutions with a continuous electrochemical scanning back and forth. There is a marked increase of the desorption rate with pH. The *pK*_a for catechol itself is 9.85 and the solubility of the reduced form will therefore be higher at higher pH due to deprotonation. No change in stability is observed between pH 9 and 11. The rate of desorption increases somewhat when the solution is stirred. The electrode is stable when stored in air.

Catalytic Oxidation of NADH at the NSCH₂ Electrode. Cyclic Voltammetry. Figure 3, curve a, shows a cyclic

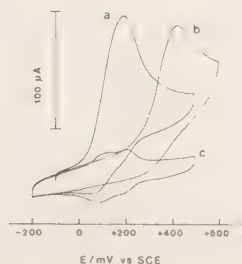


Figure 3. Catalytic oxidation of NADH mediated by adsorbed NSCH₂ (curve a), 2.3 mM NADH, pH 7.0, sweep rate 50 mV s⁻¹. Curve b shows oxidation at a naked graphite electrode in the same solution. Curve c shows NSCH₂ modified electrode in a buffer without NADH. Starting potential was -100 mV vs. SCE.

voltammogram obtained with the NSCH₂ covered electrode in a solution which was 2.3 mM in NADH. It can be seen that there is a great increase in the anodic current compared to a scan in a buffer without NADH (curve c). The reason is that NADH diffuses up to the electrode surface and reduces the NSC to NSCH₂. As NSCH₂ is regenerated by NADH during the sweep, there will be an increase in the current. For the same reason the cathodic current is smaller in the presence of NADH as NSCH₂ is produced simultaneously from both an electrochemical and a chemical step. The overall reaction thus proceeds as shown in eq 1–3. The rate-determining step



is given in eq 1 with a rate constant *k*₁. Thus the rate of the reaction in eq 2 can be considered fast compared to the one in eq 1. The overall oxidation of NADH by the electrode is given in eq 3.

The catalytic effect can be seen directly when curve a is compared with curve b in Figure 3. Curve b is obtained with an untreated graphite electrode. The decrease in overvoltage is 225 mV at pH 7.0 and 315 mV at pH 9.0.

Succeeding scans with stirring between them result in only minor decreases of the peak height of curve a. The decrease follows the course given by Figure 2 which indicates that a repeated recycling can take place without loss of activity from other sources than desorption of NSCH₂. The peak current was proportional to the NADH concentration in the range tested 0.7–7 mM. Andrieux and Saveant (24) derived a relation between the peak current and the concentration for the case when *k*₁ in eq 1 is infinitely fast.

$$i_p = 0.496nFAD_R^{1/2}v^{1/2}C_R^*(nF/RT)^{1/2} \quad (4)$$

The symbols have their usual significances. Low values of *k*₁ result in lower values of the constant (0.496). This constant was found to be 0.30 for a NSCH₂ electrode with a coverage of *Γ* = 1.3 × 10⁻⁹ mol cm⁻². The rate constant *k*₁ in eq 1 associated with this value could then be calculated to ~2 × 10⁶ M⁻¹ s⁻¹.

Rotating Disk Experiments. Experimental results with the NSCH₂ covered electrode mounted in a rotating disk assembly are shown in Figure 4. A Levich line, assuming a very fast chemical reaction, was calculated (curve a) by using a diffusion coefficient of 2.4 × 10⁻⁶ cm² s⁻¹ (25) for NADH.

The currents obtained in NADH solutions are shown by curves b–d. The potential was kept at +275 mV vs. SCE, i.e., just on the plateau obtained when the voltage was scanned

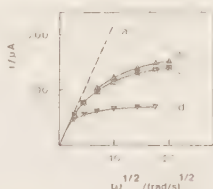


Figure 4. Anodic oxidation of NADH on a rotated disk graphite-NSC electrode. Curve a shows the theoretical line for a very fast reaction. Experimentally obtained curves are shown for a surface coverage of (b) 1.3×10^{-9} , (c) 1.5×10^{-9} , and (d) 0.5×10^{-9} mol cm $^{-2}$. Applied voltage was +275 mV vs. SCE, pH 7.0. Concentrations for a, b, and d were 1.90 mM and for c was 1.76 mM NADH.

slowly during rotation. The rate of rotation was increased stepwise. Following each step the current stabilized in a few seconds. When the rotation rate was returned to the original slower value, the current had decreased according to the desorption in Figure 2. An unmodified electrode gave no oxidation current at this voltage. The NADH concentrations in b and c were 1.90 mM and in d, 1.76 mM. Curve b was obtained with an electrode with a surface coverage of 1.3×10^{-9} mol cm $^{-2}$. The coverages for curves c and d were 1.5 and 0.5×10^{-9} mol cm $^{-2}$, respectively.

As seen in Figure 4 the current reaches a maximum level as the rotation speed is increased. This is expected in a rotating disk experiment, when the current is completely under kinetic control. Andrieux et al. (26) derived the following equation for surface modified catalytic electrodes when the current has reached its maximum value.

$$i = nFAk_1\Gamma_{\text{Ox}}C_R^* \quad (5)$$

The symbols have their usual significance and k_1 is the rate constant of eq 1. At the potential used the rate of the electrochemical reaction, eq 2, can be considered infinite and the reaction in eq 1 is the rate-determining step. In this way the curves shown in Figure 4 give k_1 values of 1.2, 1.0, and 1.4×10^6 M $^{-1}$ s $^{-1}$ which agree well with the one evaluated from the cyclic voltammetrical experiments.

Electrochemistry of Adsorbed ASCH $_2$. Cyclic voltammograms of ASCH $_2$ adsorbed on graphite electrodes were generally very similar to those of the adsorbed NSCH $_2$ except that the electrochemical reaction rate was somewhat smaller. The apparent rate constant was about 0.8 s $^{-1}$. The $E_{1/2}$ and its pH dependence were the same and there were also two peaks indicating separate one-electron steps.

The intramolecular conduction of the compound II should be smaller than that of compound I, and it was found that the electrochemical rate is at least 3 times slower than that for the naphthalene. The original coverage was about the same but the desorption was faster. The desorption was in fact too fast to make experiments with a rotating disk electrode with a constant and well-defined coverage.

Catalytic Oxidation of NADH on the ASCH $_2$ Electrode. Figure 5 shows a cyclic voltammogram obtained with ASCH $_2$ adsorbed on a graphite electrode. Curve c shows the voltammogram in a buffer without other reagents. The surface coverage was 0.7×10^{-9} mol cm $^{-2}$. In the presence of 2.4 mM NADH a catalytic oxidation occurs at (a) in the upper curve. At more anodic voltages, (b) in Figure 5, NADH is oxidized directly without involvement of the attached mediating groups.

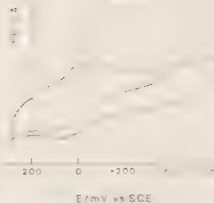


Figure 5. Catalytic oxidation of NADH mediated by adsorbed ASCH $_2$ (a) as well as direct oxidation of NADH (b). Surface coverage was 0.7×10^{-9} mol cm $^{-2}$ with 2.4 mM NADH, pH 9.0, and sweep rate 50 mV s $^{-1}$. Curve c was obtained under the same conditions except that no NADH was present. Starting potential was -200 mV vs. SCE.

A comparison between the catalytic oxidation of the NSCH $_2$ and the ASCH $_2$ electrodes (Figures 3 and 5) shows that the reaction between NADH and the mediating group is much slower for the ASCH $_2$ electrode. The overvoltage was decreased less effectively than on the NSCH $_2$ electrode. As a matter of fact the ASCH $_2$ species was synthesized in the belief that the rate of the chemical step would be favored by the more elevated position of the catechol group.

ACKNOWLEDGMENT

The authors thank Christer Westerlund, Department of Organic Chemistry 1, for valuable discussions during the synthetic work.

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RECEIVED for review April 13, 1981. Accepted July 28, 1981.

ELECTROCHEMICAL STABILITY OF CATECHOLS WITH A PYRENE SIDECHAIN STRONGLY
ADSORBED ON GRAPHITE ELECTRODES FOR CATALYTIC OXIDATION OF DIHYDRONICOTINAMIDE-
ADENINE-DINUCLEOTIDE (NADH).

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ABSTRACT

The electrochemical stability and reactivity of 4-(2-(1-pyrenyl)vinyl)catechol (PSCH2) and 4-(2-(1-pyrenyl)ethano)catechol (PECH2) strongly adsorbed on graphite electrodes were investigated as a function of the applied potential at pH 7.0.

The surface coverage of these compounds ranged from 0.1×10^{-9} to 2.7×10^{-9} mole/cm². The "modified" electrodes exhibited deactivation which could be explained by second order reactions between the catechols and the electrochemically produced quinones coupled with a second order reaction between the quinones. The ethano compound showed a much larger decay rate; probably due to the free rotation around the saturated bond connecting the pyrene part and the catechol group. The deactivation was apparently not associated with desorption of the compounds.

The catechols in the oxidized form could catalytically oxidize NADH. The overpotential for NADH oxidation was thus decreased from 410 mV to 150 mV vs SCE at pH 7.0. However, the catalytic current was found to decrease exponentially with increasing number of scans. The rate of this deactivation of the catalytic electrode was found to be inversely proportional to the coverage of immobilized mediator. The deactivation could be explained by a chemical coupling reaction between the mediator and NADH, forming a complex which gradually blocked off the surface of the electrode. The probable nature of the complex makes it unlikely that "capping" of active sites, e.g., the 2, 5 and 6 positions, on the catechol ring would effectively prevent the blocking and hence, deactivation of the catalytic electrodes.

INTRODUCTION

The coenzyme Nicotinamide-Adenine-Dinucleotide (NADH) is oxidized directly at different electrode materials only with high overvoltages¹. The overvoltage at pH 7 is about 1.1 V at a carbon² and 1.3 V at a platinum electrode. Attempts have been made to link the electrochemical oxidation of the coenzyme to the analysis of substrates for clinical purposes^{4,5}. About 300 dehydrogenases are known which are dependent on NADH or NADPH as redox transfer agents. Electrodes modified to oxidize NADH more easily should therefore open up new applications both in analysis and in biotechnology.

Surface modifications have been proposed as a means of reducing the overvoltage of the electrochemical oxidation of NADH. Blaedel and Jenkins⁶ reduced the overvoltage from 0.2 V to 0.45 V vs SCE with an electrochemical pretreatment of the electrode. They assumed that hydroxyl, carbonyl and quinone groups produced by the oxidative pretreatment of the electrode caused this decrease of overvoltage. It has been shown in this laboratory that a catalytic electrode could be produced by the covalent attachment of 1,2-hydroquinones to the surface of pyrolytic graphite⁷. That work demonstrated the possibility of producing catalytic electrodes by the covalent incorporation of electron transfer mediating groups onto the surface of the electrode. Recently, Degrand and Miller⁸ modified a vitreous carbon electrode with a polymer coating, to which dopamine was chemically bound, and showed that such electrodes were catalytically active for a number of cycles. The decrease of overvoltage was about 0.25 V.

It has been observed that polycyclic aromatic compounds adsorb strongly on graphite surfaces. The adsorption increases in strength with an increasing number of aromatic rings⁹⁻¹². Since it is known that some 1,2-quinones are

mediators in homogeneous solutions and also when covalently bonded to a surface⁷, there was a possibility that adsorbed 1,2-quinones might also mediate the electron transfer from NADH to the electrode. No such effect could be seen for 1,2-naphthaquinone, alizarin, phenanthrenequinone or for a number of other condensed aromatic quinones studied previously¹³. The adsorbed quinone functionality which probably lies flat on the surface, seems to be in an unfavorable position for electron exchange with NADH. In an earlier report¹⁴ a molecule which had a catechol group attached via a double bond to a naphthalene ring system was synthesized and studied. The small movement of the catechol group about the rigid double bond was found to be sufficient to facilitate fast electron exchange with NADH. However, the lifetime of the electrodes seemed to be limited by a relatively fast desorption of the compound.

This paper describes two types of compounds in which the naphthalene used previously was replaced with a pyrene ring system. The adsorption of this species on the electrode surface should be much stronger, since the number of aromatic rings is doubled. The solubility in aqueous solutions is also much less. This adsorbed layer should be much more stable toward desorption, and hence, should provide increased electrode stability if no other deactivating process exists.

RESULTS AND DISCUSSION

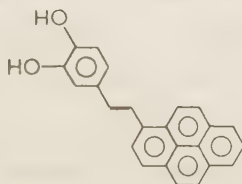
ADSORBING SURFACE AREA

Fig. 1 shows a scanning electron microscope (SEM) picture of an emery paper polished surface of the spectrographic graphite electrode. Many parallel grooves are visible with a mean separation of approximately 10 μm . In addition to these grooves from the polishing procedure, it can be seen that the surface consists of stacked flakes of differing sizes. It is obvious that the surface

area available for adsorption is much greater than the geometrical one. A calculation of the area available for adsorption is quite difficult, but the number of flakes per unit area and their mean size yields a real area estimate of two to five times the geometrical one. As mentioned in the experimental section (vide infra), the electrochemical surface area seen by a diffusing species in solution is equal to the geometrical area. This is reasonable since the diffusion layer always extends into the solution more than the electrode topography. Lack of knowledge of the exact adsorbing electrode area is a complication, but this drawback is outweighed by the material's propensity for adsorption. Actually, a surface area greater than the geometric one is an advantage when the electrochemical catalysis process proceeds through an EC mechanism. This is realized when surface coverage per adsorbing real area of a catalyst is considered. In the case where only a moderate surface coverage is achieved, the resulting projection of that coverage from a plane at the surface of the electrode as seen from the solution by a diffusing species, may be many times greater, depending on the roughness of the electrode. Hence, a high catalytic current may be achieved although the actual surface coverage may be low.

NUMBER OF LAYERS

The cross sectional area of a PSCH₂- molecule (A) when adsorbed flat



(A)

on the surface is calculated, using the geometric area, to be $0.6 \times 10^{-14} \text{ cm}^2$. For the electrode with the lowest surface coverage attained, 0.10×10^{-9} , mole/cm², this would correspond to 1/3 of a monolayer, and for the highest coverage of 2.7×10^{-9} mole/cm², the coverage would be almost 10 monolayers. The previous discussion about the dimension of the adsorbing surface area of this electrode material obviously means that the actual surface coverages are much lower.

Fig. 2 shows how two electrochemical parameters for the adsorbed PSHC2 molecule vary with coverage. The separation of the peaks, ΔE_p , a measure of the apparent electrochemical rate constant¹⁵, is seen to remain constant at 10 mV for a scan rate of 50 mV/s up to a coverage of 1×10^{-9} mole/cm². A maximum peak separation of 30 mV is reached for a coverage of 2.7×10^{-9} mole/cm². The small peak separation at low coverages is close to the theoretical value for an immobilized redox couple in equilibrium with the electrode potential¹⁶. The width at half peak height, $\Delta E_{p/2}$, also stays virtually constant, at 55 mV, up to a coverage of 1×10^{-9} mole/cm². The effect of the increased coverage causes peak broadening which is not quite as abrupt, but still clear. It has been shown in a series of papers by Laviron and coworkers¹⁵⁻¹⁸, that if there are interactions between molecules adsorbed on an electrode surface, these will affect the peak potentials and the peak widths. Therefore, the broadening of the peaks and the increasing peak potential shifts that begin around 1×10^{-9} mole/cm², strongly suggest that monolayer coverage has been reached at this value of coverage. A monolayer coverage of 1×10^{-9} mole/cm² corresponds to an adsorbing surface area of 0.84 cm^2 , which is 3 times greater than the geometrical one. This factor is quite close to the one estimated from the number and size

of the flakes. Experimental results from the catalytic oxidation of NADH also suggest that a monolayer is reached at this coverage. It is therefore concluded that the actual coverages attained in this work range from 1/10th of a monolayer up to ca. 3 monolayers.

ELECTROCHEMISTRY OF ADSORBED PSCH2

Fig. 3 shows cyclic voltammograms of a PSCH2 covered electrode obtained in a buffer solution with no NADH present. The peak current was found to increase linearly with scan rates from 25 mV/s to 200 mV/s. At 200 mV/s kinetic effects in the electron transfer rate begin to influence the linear relationship¹⁹. The apparent electrochemical rate constant was calculated to be $4(\pm 1) \text{ s}^{-1}$ by measuring the separation of the peaks as a function of the scan rate between 25 and 600 mV/s for an electrode with a coverage of $0.90 \times 10^{-9} \text{ mole/cm}^2$ at pH 7.0¹⁵. The width at half peak height is 55 mV which is close to the theoretical value $90/n$ for a two electron, two proton electrochemical reaction involving an adsorbed species¹⁶. The number of electrons in the reaction, assuming a Nerstian response, was calculated from the equation:

$$i_p = [n^2 \cdot F^2 / 4RT] \cdot A \cdot \Gamma_0 \cdot v \quad (1)$$

where the parameters have their usual significance^{8,20}. For an electrode with a coverage of $0.90 \times 10^{-9} \text{ mole/cm}^2$, a plot of v versus i_p/Q , where $Q = nFx$, and x is the number of moles, gave a value of $n = 1.97(\pm 0.05)$ for scan rates lower than 50 mV/s. Higher scan rates yielded lower n -values due to the influence of the electrochemical kinetics on the peak current (ref. 17, Fig. 3).

Fig. 4 shows $E^{0'}$ as a function of pH. The $E^{0'}$ was taken as the average value of the anodic and cathodic peak potentials at a scan rate of 25 mV/s,

where no kinetic effects would adversely distort the peaks. The slope was found to be 58 mV/pH-unit over a pH range from 2.0 to 11.0, as expected for a two electron, two proton electrochemical reaction. However, since the pK_a of a soluble catechol (pyrocatechol) is known to be 9.8,²¹ a change of slope from 58 mV/pH to 29 mV/pH would be expected at higher pHs. In addition, a change in the pK_a by the aromatic substituent would be expected to give a lower value, due to the stabilizing effect of the conjugated system on the deprotonated species. Since no change in slope was observed, it has to be assumed that no deprotonation of the adsorbed PSCH2 is taking place at the expected pH. Changes in pK_a 's of adsorbed species have previously been reported, for example, for FAD adsorbed on the same kind of electrode^{12,29}.

STABILITY OF ADSORBED PSCH2

The low solubility of PSCH2 in water and the strength of the adsorption onto the spectrographic graphite made the adsorbed layer very stable toward desorption. When an electrode with a coverage of 0.76×10^{-9} mole/cm² was washed with deionized water for one hour, no loss of adsorbed species could be detected. Even a jet of methanol directed on the electrode surface for several minutes resulted in only a few per cent loss. The experiment was performed when the mediator was in its reduced state. When the potential was scanned for extended periods of time, a slow decrease of the electroactive species was observed. After five hours of scanning (710 scans) between -200 mV and +430 mV at pH 7.0, 65% of the original coverage of 0.93×10^{-9} mole/cm² remained. However, it was found that this loss of activity was not due to desorption but was caused by a potential dependent chemical reaction of the mediator on the electrode surface. A similar potential dependence of the stability has been

observed previously in this laboratory²³ for catechols immobilized through covalent or polymeric attachment on glassy carbon electrode surfaces.

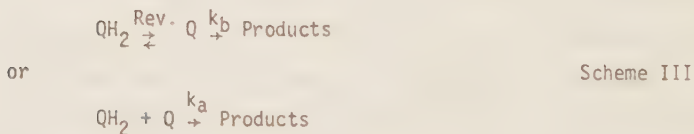
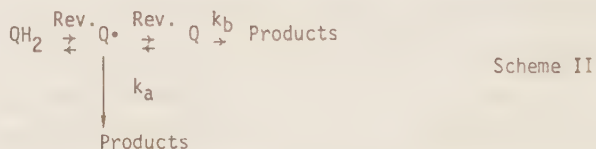
The potential dependence of the adsorbed layer was studied by applying a constant potential and then monitoring the electrochemically active mediator as a function of time. The amount of electrochemically active mediator was measured at discrete times by scanning the potential over the range required for one complete cyclic voltammogram. The scan rate was 100 mV/s, and the total scan time was 12 seconds. Since the time scale for the whole experiment was on the order of 6 to 12 hours, individual measurements of the coverage by cyclic voltammetry had a negligible influence on the measured decay rate. In order to insure that the value of the acquired rate constant was unaffected even at the lowest decay rates, only a few scans were made. The experiments were performed at pH 7.0 using eight different electrodes at eight different settings of the potential. The coverage at $t = 0$ did not vary by more than $\pm 15\%$ from a mean value of 1.9×10^{-9} mole/cm². A plot of $\log(r/r_0)$ vs time, where r is the coverage at any given time and r_0 is the coverage at $t = 0$, yielded a set of curves with an initial fast decay followed by a fairly linear segment (23). At potentials lower than 0.0 V the rate of loss is virtually zero. When the potential is made more positive, the decay rate starts to increase, and when the potential is near $E^{0'} + 95$ mV at pH 7.0, a maximum is observed. When the potential is increased further the decay rate levels off (Fig. 6).

This observed potential dependence of the stability of the adsorbed layer has at least two aspects. Firstly, it could be argued that the quinone form of the PSCH₂-molecule could have a much higher rate of desorption than the hydroquinone form. However, the presence of a peak in Fig. 6 does not support

such an argument. Secondly, the simple first order chemical process



proposed in the deactivation of an eugenol modified electrode²³ cannot alone explain the observed behavior for the PSCH2 electrode. More complicated reaction paths must exist. Examples of surface reactions which could explain the observed behavior are



where QH_2 is the reduced species, $\text{Q} \cdot$ is the semiquinone, and Q is the quinone species.

If the lifetime of the semiquinone is sufficiently long, reaction scheme II can account for the observed peak in Fig. 6. However, the sharpness of the anodic and cathodic peaks and the fact that n was calculated as 2.0 indicate that this pathway may be less likely than the reactions in scheme III.

A solution of the differential equation (see appendix)

$$-dr/dt = k_a \cdot [\text{QH}_2] \cdot [\text{Q}] + k_b \cdot [\text{Q}]^2 \quad (2)$$

describing the time dependent loss of electroactive mediator for a second order reaction between the hydroquinone and the quinone, coupled with a second order reaction between the quinones is:

$$\left(\frac{1}{\Gamma} - \frac{1}{\Gamma_0}\right) = (k_a \cdot B(E) + k_b) \cdot t / (1 + (B(E))^2) \quad (3)$$

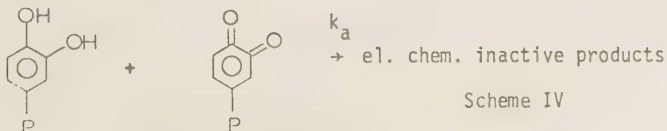
where the rates of the reactions are k_a and k_b , and the equation

$$B(E) = [QH_2]/[Q] = \text{Exp}[(E^{0'} - E) \cdot 2F/RT] \quad (4)$$

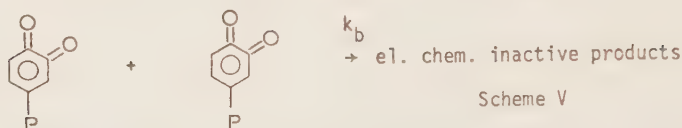
gives the relative concentrations of the two species at different potentials. Fig. 5 shows plots of $(1/\Gamma - 1/\Gamma_0)$ vs. time, t . These plots, all passing closely through the origin, affirm the proposed second order nature of the decay mechanism. The potential dependence of the slopes in Fig. 5 is shown in Fig. 6. The trace in this figure is drawn using the equation

$$k_{\text{exp}} = (k_a \cdot B(E) + k_b) / (1 + (B(E))^2) \quad (5)$$

which is the "slope-factor" in equation (3). A good fit between the experimental data points and the theoretically calculated solid trace in Fig. 6 is obtained when $k_a \approx 10 \cdot 10^5 \text{ cm}^2 \text{ mole}^{-1} \text{ sec}^{-1}$ and $k_b \approx 0.8 \cdot 10^5 \text{ cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$. Since the hydroquinone form is a slightly better nucleophile than the quinone, the reaction rate k_a of the chemical reaction

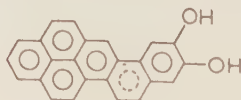


would be expected to be somewhat faster than the rate k_b of the reaction



In both cases, ether linkages and other unspecified polymeric forms may be produced, decreasing the concentration of electroactive catechols on the surface²⁴. However, a curve derived from the radical reaction (II) would fit just as well, assuming that the radical reaction is 5 times faster than the quinone one and that the $E^{0'}$ of the two steps are equal. It is not possible from the acquired data to distinguish between these two models; both reactions may actually occur simultaneously.

Other reactions also occur. If the potential is scanned to +1.24 V, a large oxidation peak is seen as the background discharge becomes prominent. This peak is most likely due to the oxidation of the pyrene ring²⁵. When the potential scan is reversed, the redox couple at +95 mV is no longer seen and a new one appears at -82 mV. This redox couple shows a pH dependence that can be ascribed to a two electron, two proton reaction. The stability of the new redox couple is independent of time, and remains unaffected after several hours of scanning. The low $E^{0'}$ value and its inability to catalyze NADH makes it probable that the redox couple is formed through cyclization of the pyrene and catechol parts of the molecule (B). The dotted circle in structure (B) indicates a new six-membered ring, formed upon cyclization of compound (A).



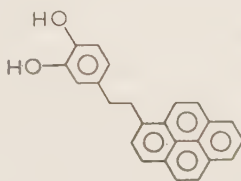
(B)

The cation radical formed via oxidation of the pyrene ring²⁵ would make it possible for the molecule to cis-trans isomerize at the otherwise rigid double bond. The formation of a new redox couple is seen also for the PECH2 modified electrode. The yield of the new compound is not quantitative and seemed to be a function of coverage.

Another "bound" mediator, 4-(2-(9-anthracene)vinyl)catechol, not further investigated in this work, showed the same disappearance of the redox couple after oxidation at +1.25 V, but no new redox couple was found at -82 mV. A probable reason is, for conformational reasons, this species cannot form another six membered ring through cyclization.

ELECTROCHEMISTRY OF ADSORBED PECH2

Fig. 7 shows cyclic voltammograms of a PECH2 (C) covered electrode, obtained in a PECH2 free buffer solution. The $E^{0'}$ is virtually



(C)

the same as for the PSCH2 modified electrode. No significant difference in the $E^{0'}$ value would be expected with the change from a conjugated to an unconjugated "bridge" between the pyrene and the catechol ring²⁶. The same dependence of $E^{0'}$ on pH is also found. However, the electroactive coverage decreases to 50% of the original in as few as 80 cycles, when scanned between -200 and +430 mV at 50 mV/s. The PSCH2 modified electrode scanned over the same potential

range, showed only a 5% decrease for the same number of scans. Experiments, in which the electrode was washed with deionized water and methanol, showed results similar to those of the PSCH₂ covered electrode. An analogous dependence of stability on the potential was also observed. However, since the rate of the electrochemical decay was so rapid, approximately the same as the time of a single scan, it was not possible to study the rate of loss of electroactive mediator at different potentials. It is estimated there is a 10 fold decrease in stability compared to PSCH₂. This very significant change in stability must be caused by the presence of free rotation between the anchor part and the catechol in the PECH₂ molecule. Therefore, the probability of reacting with a neighbor is much greater than for the PSCH₂ molecule, in which the catechol is held in a plane by the rigid double bond. Although catalytic oxidation of NADH was observed, the immobilized mediator itself showed sufficient instability that no further investigation of the PECH₂ covered electrode was undertaken.

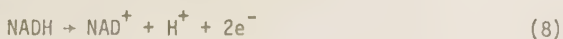
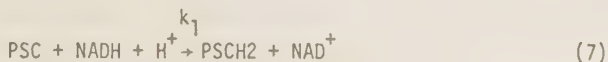
HOMOGENEOUS SIDE-REACTION OF PECH₂

In early studies on the adsorption of FAD and several flavines on spectrographic graphite electrodes^{12,27}, it was found that the electrochemical kinetics of the adsorbed species were much faster when HCl was added to the solution from which they were adsorbed. On the other hand, when PSCH₂ was adsorbed from a MeOH/H₂O/HCl solution only adverse effects were observed. After a few days in the acid solution a new redox couple started to appear on the cyclic voltammograms and after one week at room temperature no PSCH₂ was detected. The new redox couple showed a pH dependence of $E^{0'}$ identical to that PECH₂,

but the rate of decay versus the number of scans was much faster. In fact the rate of decay was about the same order of magnitude as found for the PECH₂ modified electrode discussed above. This strongly suggests that the new redox couple, formed slowly in MeOH/H₂O/HCl solution, is a species in which an addition to the double bond has occurred. Some of the species in the solution that could be responsible for such an addition are HCl, added to the double bond, and MeOH or water added by acid catalysis. Considering the positive change in the $E^{0'}$ value from +95 mV to +180 mV vs SCE, a species formed from the HCl seems likely²⁶. The electronegative chlorine atom would probably exert an electron withdrawing effect on the catechol ring. The magnitude of the change in $E^{0'}$ is surprisingly large²⁶. Further investigation of the exact nature of this new redox couple was not pursued since it showed lower stability and had a more positive $E^{0'}$ value which would be undesirable for NADH catalysis.

CATALYTIC OXIDATION OF NADH

Fig. 8, curve A, shows a cyclic voltammogram obtained with a PSCH₂ covered electrode in a 2.0 mM NADH solution at pH 7.0. There is a significant increase in the current at the potential where PSCH₂ is oxidized, compared to the scan in the buffer solution without NADH, curve B. The cause of the increased current is the NADH present in the solution diffuses toward the electrode and reduces the PSC produced electrochemically. The overall reaction proceeds according to an EC catalytic mechanism, equations 6-8:



The rate determining step is reaction 7, with a rate constant, K_1 .

The net electrode reaction is given by eq. 8. The catalytic peak is found at +150 mV, compared to the uncatalyzed value of +410 mV¹⁴. Thus, a decrease in the overvoltage of 260 mV is observed.

The peak current, i_p , was found to vary only slightly with the coverage of the electrode for the first scan and followed quite closely the equation given by Andrieux and Saveant²⁸:

$$i_p = 0.496 \cdot nFA \cdot (D_R \cdot nFv/RT)^{1/2} \cdot C_R \quad (9)$$

For the two lowest coverages used, 0.10×10^{-9} and 0.21×10^{-9} mole/cm², slightly lower values than 0.496 were observed. From Fig. 1 in the paper by Andrieux and Saveant, where a plot of the "constant" 0.496 is given as a function of

$$\log [K_1 \cdot \Gamma / (D_R \cdot nFv/RT)^{1/2}] \quad (10)$$

the reaction rate constant, K_1 , was calculated to be $1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. This is 10 times faster than that calculated previously²⁹ for a naphthalene-vinylcatechol adsorbed on a graphitic surface¹⁴.

The homogeneous rate constants have been calculated previously for several catechols with different sidechains⁷. The rate constants were found to vary from 4×10^2 to $8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for 1,2-napthaquinone-4-sulfonic acid and 3,4-dihydroxybenzylamine, respectively, and to be a function of ΔG in a homogeneous solution⁷. On the other hand, 1,2-napthaquinone showed no catalytic effect

when adsorbed on a graphite electrode, in spite of an appreciable reaction rate in homogeneous solution. Therefore, the accessibility of the quinone group as well as the ΔG of the reaction must influence the heterogeneous reaction rate.

CATALYTIC STABILITY

Despite the high stability of the PSCH2 modified electrode in buffer solution with no NADH present, consecutive scans with this electrode in a 2.0 mM NADH solution resulted in a drop of the catalytic current. The catalytic stability was found to be dependent on the original coverage of mediator. Seven different electrodes, with coverages ranging from 0.10×10^{-9} to 2.7×10^{-9} mole/cm² were studied.

Fig. 9 shows the decrease in the catalytic peak current expressed as a ratio of $i_{p,cat}/i_{p,cat,1st\ scan}$ versus the number of scans. The electrodes with low coverages showed a much faster loss of catalytic activity than those with high coverages. For an electrode with a coverage of 0.10×10^{-9} mole/cm², only 50% of the catalytic current remained after 5 scans. The electrode with the highest coverage, 2.7×10^{-9} mole/cm², exhibited a much higher degree of stability and more than 90% of the catalytic current remained after 30 scans. The exponential character of the curves in Fig. 8 is verified by the linearity of the plots of the logarithm of $i_{p,cat}/i_{p,cat,1st\ scan}$ versus the number of scans. A set of straight lines is obtained, with different slopes depending on the coverage, suggesting a first order deactivation reaction. In fact, the curves drawn in Fig. 9 are obtained from the regression analysis of the logarithmic plots. The decay follows the equation:

$$i_{p,cat} = i_{p,cat,1st\ scan} \cdot 10^{(-k \cdot (n-1))} \quad (11)$$

where k is the slope and n is the number of scans. An inverse relationship was found to exist between the slope constant, k , and the coverage, Γ . This is shown in Fig. 10, where $1/k$ is plotted versus Γ , giving a linear relationship for coverages below 1×10^{-9} mole/cm². However, a marked deviation from a linear dependence is observed for the two highest coverages. According to previous discussion, these coverages correspond to two or three monolayers. The deviation indicates that an apparently more stable catalytic electrode is produced per adsorbed mediator and appears to be a side effect of the deactivation process. This will be discussed later.

INFLUENCE ON THE MEDIATOR BY THE CATALYSIS

The adsorbed mediator was studied using cyclic voltammetry before and after the catalytic NADH oxidation experiments. The filled circles in Fig. 2 show the peak shifts, ΔE_p , and the peak widths at half peak height, $\Delta E_{p/2}$ as a function of the coverage after the catalytic oxidations. The peak separations seem to be a function only of the electrode coverage. This indicates that there has been no change in the electrochemical kinetics induced by the catalytic NADH oxidations¹⁶. The peak widths, however, show a marked change, giving much broader waves after catalysis (Fig. 2). This effect increases with decreased coverages. The broadening of the waves suggests that stronger interactions between the adsorbed mediators occur after several catalytic scans have been made¹⁶.

The data in Table A show that a noticeable decrease in the electroactive mediator has taken place after the catalytic oxidations of NADH. This decrease may be due to either loss of mediator into the solution or deactivation of the adsorbed species. Table A also gives the total amount of oxidized NADH produced by each electrode, ΣNADH . The change in the coverage of the mediator,

$\Delta\theta$, is also given. The ratio of $\Sigma\text{NADH}/\Delta\theta$ gives the total number of oxidized NADH molecules per inactivated or lost mediator. For coverages below 1×10^{-9} mole/cm² a constant value of $\Sigma\text{NADH}/\Delta\theta = 1090 (\pm 30)$ is obtained. This shows that, on the average, 1100 NADH molecules can be oxidized by one adsorbed mediator, before a sidereaction with NADH makes it electrochemically inactive.

BLOCKING MECHANISM

Even though a decrease in the coverage of electroactive mediator is observed after successive scans in a NADH solution, the amount of mediator remaining on the electrode should be sufficient to maintain the catalytic current with very little decrease on the subsequent scan (see data in Table A). The fact that a decrease is actually observed, suggests that a "blocking" of the available mediators left on the electrode is occurring. Adsorption of the product NAD^+ is ruled out as a blocking mechanism²³, since experiments in which the electrode was dipped into a 2.0 mM NAD^+ solution for several minutes showed no adverse effects on the catalytic current. Thus, the experiments give evidence that there is a chemical side-reaction between the oxidized PSCH₂ adsorbed on the electrode and the NADH in the solution, and that the reaction complex is at most, only partly desorbed.

A mechanism explaining the experimental data for the catalytic behavior is proposed by the following model. The blocking is caused by a reaction between the adsorbed PSC and the NADH in the solution. The probability of a side-reaction occurring when one PSC molecule oxidizes one NADH is on the average 1/1100, as indicated by the data of Table A. When the side-reaction takes place, part of the electrode surface is blocked from participating in further catalytic oxidation. Since the cross section of one NADH (2×10^{-14} cm²) is approximately 4 times³¹ that of a PSCH₂ molecule (0.6×10^{-14} cm²),

several adsorbed mediators will become unavailable for NADH catalysis when a NADH molecule is coupled to the surface as illustrated in Fig. 11. However, the mediators which are screened from reaction with solution NADH are still electroactive, as evidenced by the i -E wave on a scan in a buffer solution. This explains why a modified electrode, after scanning in a NADH solution, shows catalytic decay in spite of the fact that the coverage appears to be sufficient for a maximum current.

The difference in stability for electrodes with varying coverages can be explained by the fact that an electrode with low coverage turns over a significantly larger number of NADH molecules per adsorbed mediator than an electrode with a high coverage. As a consequence, the probability for a side-reaction is much greater.

Table B gives the total number of oxidized NADH molecules turned over by the adsorbed PSCH₂, NTC. Obviously, the numbers of turnovers versus coverage decreases with increasing coverage thereby making the probability for a side-reaction to occur much smaller. Table B also gives the probability for side-reaction, NTC/M, and hence, the average number of oxidations necessary for deactivation to occur. The percent decrease in the catalytic current versus the total number of scans (Fig. 9) is given as PDCC. The conspicuous compatibility between the probability, NTC/M, and the percent decrease, PDCC, strongly suggests that there is a correlation between the fraction of mediators deactivated and the fraction of catalytic current lost. This correlation may be explained by a model in which the surface area of the electrode is divided into a discrete number of sites; each is the size of one NADH cross section. Every time a NADH molecule is coupled to a mediator on the surface, one of these sites

is no longer available for catalysis. The larger the number of coupled NADH molecules, the greater is the area that is no longer available for catalysis. This model also infers, that at each NADH site in which blockage has occurred, no more coupling reactions can take place. This means that only one mediator on the average, per NADH cross section is deactivated. This one to one correlation would account for the compatibility between the probability NTC/M and the percent decrease (PDCC) given in Table B.

As discussed in "CATALYTIC STABILITY", the apparent stability seemed to be better for electrodes with coverages higher than 1×10^{-9} mole/cm². It was also concluded in "NUMBER OF LAYERS", that coverages greater than this corresponded to more than a monolayer. On the other hand, the data in Table B and Fig. 10 suggest that the number of NADH oxidations per mediator necessary for a side-reaction to take place decreases from 1100 to below 600, actually indicating a less stable electrode. This seemingly contradictory behavior is quite compatible with the blocking mechanism proposed above.

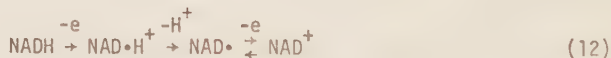
As two or three layers build up, the strength of adsorption for these layers decreases compared with mediators adsorbed directly to the surface. The strength of adsorption is decreased to such a degree that the PSC-NADH complex is no longer retained on the surface of the electrode, but is lost to the solution. This behavior is consistent with the data presented in Fig. 2. The figure shows that there is virtually no change in $\Delta E_{p/2}$ for the multilayer coverages, whereas the submonolayer covered electrodes showed significantly broader peaks. The broader peaks are evidence for stronger interactions between the different species on the surface of the electrode¹⁶. However, as more mediators per unit area are made available for catalysis, they are also

made available for side-reactions. Thus, the accumulated possibility for a side-reaction per unit NADH site increases, since the protective cover of the complex is desorbed, leading to an apparently lower number of oxidations necessary for a side-reaction to occur.

COUPLING REACTION

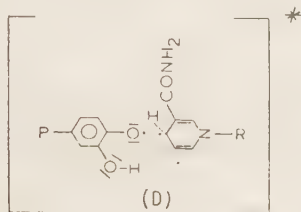
When a PSCH2 modified electrode ($\Gamma = 0.7 \times 10^{-9}$ mole/cm²) was scanned several times in a 2.0 mM NAD⁺ solution prior to scanning in a 2.0 mM NADH solution, there were no adverse effects on the catalytic current. Thus, no part of the NAD⁺ species reacts or couples to the oxidized mediator, so the coupling reaction with NADH must be a consequence of the detailed interaction between PCS and NADH in the chemical reaction mechanism.

It has been proposed, using direct electrochemical methods^{3,32}, that the direct oxidation of NADH at solid electrodes proceeds according to a mechanism given in equation 12:

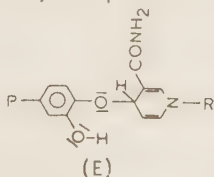


NAD[•]H⁺ and NAD[•] are intermediate radicals in an ECE mechanism. Homogeneous disproportionation between the two radical species has been also proposed. Optical methods used in connection with electrochemical experiments also provided support for this reaction path³³. It is therefore reasonable to assume that the detailed mechanism of the "catalytic" reaction between the mediator PSC and NADH involves radical species. A mechanism analogous to this was recently proposed by Miller et al.^{34,35}, in the catalytic oxidation of NADH through electrogenerated diimines. They suggested that the reaction with NADH proceeded by the rapid transfer of the 4-hydrogen in NADH to the oxidant, possibly in a stepwise fashion. It is probable that the only difference between the above

two mechanisms is that in the case of catalysis, there is a species present, namely the quinone anion radical, which acts as a strong base; i.e., the proton is abstracted at a much faster rate than it is lost to the solution when no strong base is present. A probable reaction intermediate is therefore:



where the left species is the semiquinone and the right one the $\text{NAD}^\bullet$ radical, which has been documented¹ in the electrochemical reduction of NAD^+ . In most cases the products in the next step are the catechol and the NAD^+ , but statistically, once in 1100 oxidations (Table B) the product could be a complex such as:



This molecule would account for a PSC-NADH species that poisons and "blocks off" the surface of the catalytic electrode. This species would also explain the loss of electroactivity in the adsorbed PSCH₂ molecule, observed after several oxidative cycles. The fact that the $\text{NAD}^\bullet$ radical is prone to dimerize is well established in the literature^{1,36}. A coupling reaction between the semiquinone radical and the $\text{NAD}^\bullet$ radical is therefore reasonable.

As a consequence of the structure of the proposed complex between the quinone and NADH, it seems unlikely that "capping" of sites in the quinone, e.g., the 2, 5 and 6 position by methyl groups, would have any significant

effect on the blocking reaction²³. It is therefore concluded that the catechol group probably does not offer the possibility of producing a stable catalytic electrode for NADH oxidation. Other mediators immobilized on carbon surfaces may provide a means of solving this problem^{37,38}.

EXPERIMENTAL

SYNTHESIS

4-(2-(1-pyrenyl)vinyl)catechol (pyrene-stilben-catechol = PSCH2) and 4-(2-(1-pyrenyl-ethano-catechol (pyrene-ethano-catechol = PECH2) were synthesized in a series of steps.

1. 3,4-METHYLENEDIOXY-BENZYLCHLORIDE

was obtained by adding slowly while stirring 6.55 g of 3,4-methylenedioxy-benzyl-alcohol to a solution of dry ether mixed with 10.8 g PCl_5 and 4.3 g CaCO_3 . The temperature was kept at 0°C for 10 minutes, after which the solution was carefully washed several times with ice cold³⁹ saturated Na_2CO_3 . The ether phase was then dried with anhydrous MgSO_4 while stirring for several hours, after which the ether phase was evaporated. The residual 7.3 g was identified by IR-spectroscopy and not further purified, because of its high reactivity. The yield was nearly quantitative.

2. 3,4-METHYLENEDIOXY-BENZYLTRIPHENYL-PHOSPHONIUMCHLORIDE

A mixture of 7.3 g 3,4-methylenedioxy-benzylchloride and 13.4 g triphenyl-phosphine was refluxed at 140°C while gently stirring in dry xylene for 24 hours⁴⁰. The white crystalline precipitate was filtered off and washed first with cold xylene and then with petroleum ether (boiling range $40-60^\circ \text{C}$). The product, 13.9 g (yield = 65%), was identified with IR and was not further purified.

3. 3,4-(2-(1-PYRENYL)VINYL)METHYLENEDIOXY-BENZENE

A mixture of 5.3 g 1-pyrenealdehyde and 10.0 g 3,4-methylenedioxy-benzyltriphenyl-phosphoniumchloride were dissolved in 100 ml methylene chloride. To this solution was added 100 ml of NaOH/H₂O, 50/50 (w/w), during vigorous stirring⁴¹. After 25 minutes the stirring was stopped and the reaction mixture poured into 0.5 l ice water. After washing with another 0.5 l water, the methylene chloride phase was separated and dried over anhydrous MgSO₄ for several hours. The triphenyl-phosphineoxide formed stoichiometrically was removed by adding 100 ml ethanol (99.5%) and then the methylene chloride was evaporated off. The triphenyl-phosphineoxide remained dissolved in the resulting solution, while the 1-pyrene-stilben-methylenedioxy-benzene precipitated quantitatively. The precipitate was analyzed with thin layer chromatography and found to consist of two species with a ΔR_f of 0.08. A mixture of 25/75 CH₂Cl₂/Petroleum ether (40-60° C) was used as eluent on silica gel thin layer plates. IR-spectroscopy showed only the presence of the stilbene species, but NMR indicated that a 50/50% mixture of the cis and trans forms were present. The trans was separated quantitatively from the cis by first adding 25 ml CH₂Cl₂ and then, while slowly stirring, adding petroleum ether. After 24 hours at +4° C, greenish-yellow, slightly fluorescent, needle-like crystals were filtered off. Thin layer chromatography showed the presence of one species, which was identified with NMR as the trans form. Yield of the trans form was 2.5 g which corresponds to 31%. Total yield of cis and trans was 5.5 g which corresponds to 68%. Only the trans form was used for further synthesis. Elemental analysis gave: C = 86.5%: H = 4.5%: O = 8.8% (theoretical 86.2, 4.6, 9.2).

4. 4-(2-(1-PYRENYL)VINYL)CATECHOL

1.0 g 3,4-(2-(1-pyrenyl)vinyl)methylenedioxy-benzene was dissolved in 75 ml dry CH_2Cl_2 in a nitrogen atmosphere. Through a septum with the use of a syringe, 0.5 ml of 1.0 M BBr_3 dissolved in hexane was added. The temperature was kept below -65°C with an ethanol dry-ice bath. After 30 minutes the reaction mixture was poured into a 1.0 L solution of water and ice. Nitrogen was bubbled continuously through the water-ice bath for another 30 minutes while stirring. The methylene chloride phase was extracted and dried with anhydrous MgSO_4 . In the drying process the MgSO_4 became strongly colored, probably due to adsorption of polymeric products formed in the reaction⁴². The described procedure varied slightly from the one proposed by Eisenbaum et. al.⁴³ in the cleavage of aryl-methyl-ethers. After drying, the solvent was evaporated until only 20 ml remained, after which 20 ml petroleum ether was added. The solution was cooled down to -30°C and a black undefined precipitate was filtered off. The temperature was lowered to -50°C where bright yellow flakes started to precipitate. These flakes were filtered and dried in a vacuum dessicator for 18 hours during which the powder turned slightly green. The powder was then stored in a dry nitrogen atmosphere. The product was identified as the desired product by mass spectroscopy. Recovered amount was 0.6 g; yield = 62%. A better yield might have resulted from using BCl_3 instead of⁴⁴ BBr_3 . Elemental analysis: C = 85.7%; H = 4.7%; O = 9.6% (theor. 85.7, 4.8, 9.5).

5. 3,4-(2-(1-PYRENYL)ETHANO)METHYLENEDIOXY-BENZENE

was synthesized from 3,4-(2-(1-(pyrenyl)vinyl)methylenedioxy-benzene by hydrogenation. 1.0 g of the vinyl compound was dissolved in 50 ml ethylacetate. To this solution was added 0.20 g 10% Pd on activated carbon, and hydrogen at a

pressure of 1 atmosphere was applied⁴⁵. When the reaction was stopped after 24 hours of agitation, several spoons of Celite Filter Aid were added to the mixture in order to be able to filter off the Pd catalyst without igniting the solvent. The remaining solution was completely colorless, as opposed to the original strongly fluorescent greenish-yellow one. The solvent was then dried with MgSO_4 and evaporated. The recovered product, a white powder, was identified by IR and NMR. The yield was quantitative. Elemental analysis: C = 85.3% : H = 5.2% : O = 8.8% (theor. 85.7, 5.1, 9.1).

6. 4-(2-(1-PYRENYL)ETHANO)CATECHOL

was synthesized from 0.86 g 3,4-(2-(1-pyrenyl)ethano)methylenedioxy-benzene by treatment with BBr_3 as described in step 4 for the vinyl compound. After evaporation of the solvent (until only 20 ml remained) 70 ml of petroleum ether was added. The solution was stored at -15°C overnight. A white powder was filtered off and stored in a dry nitrogen atmosphere. The compound was identified using IR-spectroscopy. The spectrum matched closely the analogous vinyl compound. Recovered amount was 0.52 g, which corresponds to a yield of 63%. Elemental analysis: c = 85.3% : H = 5.4% : O = 9.7% (theor. 85.2, 5.3, 9.5).

HPLC-PURIFICATION

When the two different catechols were analyzed using HPLC, small impurities were found. To avoid complications in the electrochemical experiments, HPLC was used to produce the highly purified solutions from which the compounds were adsorbed. The chromatograph was a Chromatronix 3100. A head pressure of 1400 psi was applied from a nitrogen cylinder. For the HPLC separation, the catechols were dissolved in $\text{CH}_2\text{Cl}_2/\text{EtOH}$ mixtures. Typically 100 μl of the catechol solutions were injected onto the column, (Hypersil-5 μM ODS-C18, length

25 cm, i.d. 5 mm). The mobile phase used in the separations was a 88/12% mixture of ethanol and water. A few hundred μ l of the purified compounds were collected at the exit of the UV-detector, at a time coinciding with the chromatographic peak maximum. The solution was then stored at -15° C or used immediately.

ELECTRODES

The electrodes were spectrographic graphite rods, 6.0 mm o.d. (RW 003 Ringsdorff-Werke, GMBH). They were polished with emery paper (Tufbak Durite Waterproof Paper Closecote 600-A Type 1) which was placed on a rotating disc polishing machine. The polishing was performed under a continuous flow of deionized water until the end of the rod was completely flat. The electrodes were typically 3 cm long and all were cut from the same graphite rod in order to avoid batch variations. Before mounting the electrodes in the holder for the electrochemical experiment, a heat shrinkable polyethylene tube with a wall thickness of 0.1 mm was slipped over the rod in order to avoid electrical contact between the sides of the electrodes and the solution. This was done from the end opposite the polished surface to avoid affecting it mechanically or chemically. The edge of the tube was in-line with the electrode surface. The electrochemical surface area was identical to the geometric one when measured in a 2.0 mM ferrocyanide solution. The polyethylene tube as well as the sides of the electrode were quite hydrophobic, and no creeping of solution between the electrode and the polyethylene tube was observed.

MEASUREMENTS

A triangular wave with linear ramp produced by an operational amplified circuit was fed to a three-electrode potentiostat, and the cyclic voltammograms were recorded on a Houston XY-recorder model 2000. The reference electrode

was a saturated calomel electrode, SCE, Radiometer K-401, and the auxilliary electrode was a Pt wire chemically isolated from the bulk of the solution with a glass frit. All measurements were made in buffers made from 0.10 M pyrophosphate (pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) or 0.10 M phosphate (pH 2.0, 3.0, 10.0 and 11.0). Measurements of areas on the chart paper, corresponding to a quantity of electrical charge, were made with a planimeter equipped with a magnifying glass (Gelman Instrument Co.). The uncertainty in charge was estimated as $\pm 2\%$.

ADSORPTION PROCEDURE

The electrodes were modified with 4-(2-(1-pyrenyl)vinyl)catechol or 4-(2-(1-pyrenyl)ethano)catechol by dipping them into a solution which had a concentration of approximately 0.05 mM of either compound. The solutions were slowly stirred during the adsorption process. By varying the time an electrode was in contact with the solution containing the electroactive species from 1 minute to 25 minutes, the coverage could be controlled from 0.10×10^{-9} mole/cm² to 3×10^{-9} mole/cm². An upper limit of 4×10^{-9} /cm² was reached even when the compounds were adsorbed from a more concentrated solution. When the electrodes were removed from the solution in which the adsorption took place, they were immediately washed with a jet of deionized water for at least one minute.

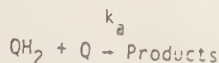
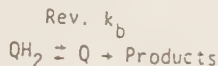
A rubber tube used in an earlier work¹⁴ in which 4-(2-(2-naphthyl)vinyl)catechol was adsorbed in a similar manner was found to be unsuitable. The compounds used in this work were quite soluble in the rubber, and the thick walls (1.3 mm) quickly adsorbed them. Therefore, the much thinner polyethylene tube mentioned above was used. Care was still taken not to dip the tube-covered electrode into the solution containing the catechols any deeper than absolutely necessary.

ACKNOWLEDGEMENTS

The authors thank Dr. C. Ueda for valuable discussions during the course of this work and Dr. J. Lyga and Dr. T. Ingler for useful ideas in the synthetic work. The authors also want to thank Dr. Charles Miller for taking the electron microscope pictures of the spectrographic graphite. Financial support is gratefully acknowledged from Kemiska Institutionen, University of Lund, Knut and Alice Wallenbergs stiftelse, Stiftelsen Bengt Lundquists Minne, (Sweden), and the National Institutes of Health, (USA), grant no. NIH GM 19181.

Appendix

The integration of the differential equation (2), A-1 (corresponds to eq. 2, p. 10) describing the surface reactions



is straight forward

$$-\frac{d\Gamma}{dt} = k_a \cdot [\text{QH}_2] \cdot [\text{Q}] + k_b \cdot [\text{Q}]^2 \quad (\text{A-1})$$

Assuming a reversible electrochemistry, i.e.



$$\Rightarrow E = E^{o'} - \frac{RT}{2F} \ln \frac{[\text{QH}_2]}{[\text{Q}]} ; E^{o'} \text{ contains the } \ln [\text{H}^+] \text{ term}$$

$$\Leftrightarrow \frac{[\text{QH}_2]}{[\text{Q}]} = \exp(E^{o'} - E) \frac{2F}{RT} = B(E) \quad (\text{A-2})$$

it is also assumed that

$$\left\{ \begin{array}{l} \Gamma_o = [\text{QH}_2]_o + [\text{Q}]_o ; t = 0 \\ \Gamma = [\text{QH}_2] + [\text{Q}] ; t > 0 \end{array} \right. \quad (\text{A-3})$$

"eliminating" $[\text{QH}_2]$ from eq. A-1 using eq. A-2

$$\Rightarrow -\frac{d\Gamma}{dt} = k_a [\text{Q}]^2 \cdot B(E) + k_b \cdot [\text{Q}]^2 \quad (\text{A-4})$$

$$\Leftrightarrow -\frac{d\Gamma}{dt} = (k_a \cdot B(E) + k_b) [\text{Q}]^2 \quad (\text{A-5})$$

from eq. A-2 and A-3 it follows

$$\Gamma = (1+B(E)) \cdot [\text{Q}] \Leftrightarrow [\text{Q}] = \frac{\Gamma}{(1+B(E))}$$

"eliminating" $[Q]^2$ from eq. A-5

$$\Rightarrow - \frac{d\Gamma}{dt} = (k_a \cdot B(E) + k_b) \cdot \frac{\Gamma^2}{(1+B(E))^2} \quad (A-6)$$

$\Rightarrow$

$$- \frac{d\Gamma}{\Gamma^2} = \frac{(k_a \cdot B(E) + k_b)}{(1+B(E))^2} \cdot dt \quad (A-7)$$

Integration yields,

$$\text{Left side ; } - \int_{\Gamma_0}^{\Gamma} \frac{d\Gamma}{\Gamma^2} = - \left[-\frac{1}{\Gamma} \right]_{\Gamma_0}^{\Gamma} = \frac{1}{\Gamma} - \frac{1}{\Gamma_0} \quad (A-8)$$

$$\text{Right side ; } \frac{(k_a B(E) + k_b)}{(1+B(E))^2} \int_0^t dt = \frac{(k_a B(E) + k_b)}{(1+B(E))^2} \cdot t \quad (A-9)$$

$\Rightarrow$

$$\left(\frac{1}{\Gamma} - \frac{1}{\Gamma_0} \right) = \frac{(k_a B(E) + k_b)}{(1+B(E))^2} \cdot t \quad (A-10)$$

which is equation 3 on page 10.

The significance of the symbols is apparent from the text or they have their usual meaning.

TABLE A

Electrode	θ_A $\times 10^9/\text{mole}$	θ_B $\times 10^9/\text{mole}$	$\Delta\theta$ $\times 10^9/\text{mole}$	ΣNADH $\times 10^7/\text{mole}$	$\Sigma\text{NADH}/\Delta\theta$	No. of scans
1.	0.028	---	---	---	---	5
2.	0.0581	0.0303	0.0278	0.0304	1093	10
3.	0.120	0.0405	0.0755	0.0855	1132	30
4.	0.158	0.0776	0.0804	0.862	1072	30
5.	0.267	0.180	0.0870	0.921	1059	30
6.	0.564	0.391	0.173	1.13	653	30
7.	0.751	0.527	0.224	1.17	522	30

TABLE A. θ_A is the amount of electroactive mediator on the electrode before scanning in a 2.0 mM NADH solution from -200 mV to +430 mV vs SCE. θ_B is the amount of electroactive mediator left after a number of catalytic scans, see last column. ΣNADH is the total amount of NADH oxidized over the total number of scans and $\Sigma\text{NADH}/\Delta\theta$ is the number of oxidized NADH molecules per deactivated mediator on the surface of the electrode.

TABLE B

Electrode	NTC	NTC/M	PDCC	(NTC/M-PDCC)	No. of scans
1.	551	0.51	0.47	0.04	5
2.	518	0.48	0.52	-0.04	10
3.	713	0.66	0.68	-0.02	30
4.	536	0.49	0.58	-0.09	30
5.	353	0.32	0.44	-0.12	30
6.	188	0.17	0.21	-0.04	30
7.	142	0.13	0.09	0.04	30

TABLE B. NTC = Number of turnovers of NADH per adsorbed mediator.

NTC/M = NTC divided by the number of NADH oxidations per mediator necessary to deactivate one of them. See Table A, Column $\Sigma \text{NADH}/\Delta\theta$.

PDCC = The percent decrease of catalytic current for the total number of scans in a NADH solution, see Fig. 9.

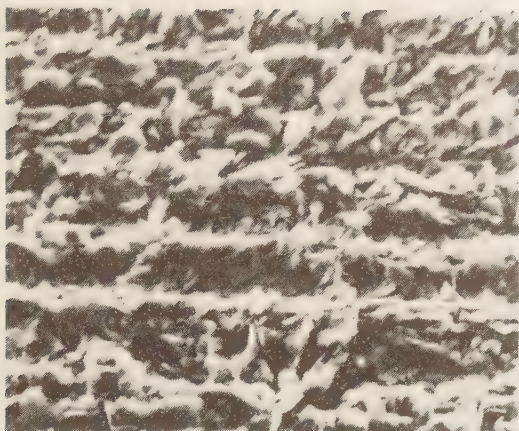


Fig. 1 A picture of an emery paper polished electrode taken with a scanning electron microscope reveals that the structure of the surface is quite complex, but still highly ordered. Besides the parallel grooves (arising from the polishing procedure) separated by a mean distance of approximately 10 μm , the surface is seen to consist of a large number of stacked parallel flakes. Magnification x560; tilt 37°.

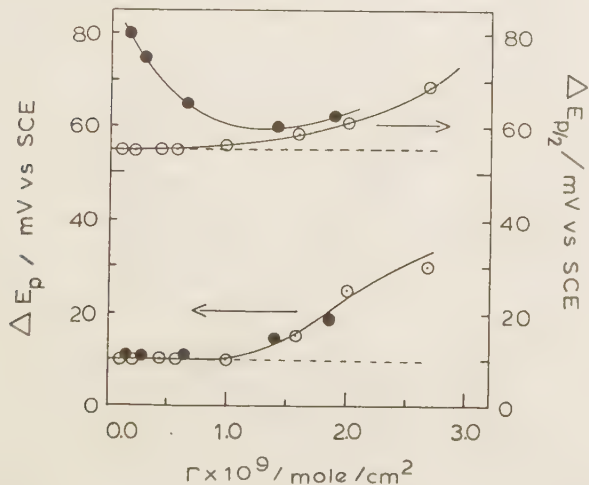


Fig. 2 The separation of the anodic and cathodic peaks, ΔE_p , and the width at half peak height, $\Delta E_{p/2}$, are shown as a function of surface coverage. The unfilled circles are before NADH oxidation and the filled ones after NADH oxidation. Both parameters are seen to be independent of the coverage below $1 \times 10^{-9} \text{ mole/cm}^2$, but start to increase at higher coverages. The data points for a coverage of $1.58 \times 10^{-9} \text{ mole/cm}^2$ were taken separately from the others which were also used for NADH oxidations. The scan rate was 50mV/s. The pH was 7.0.

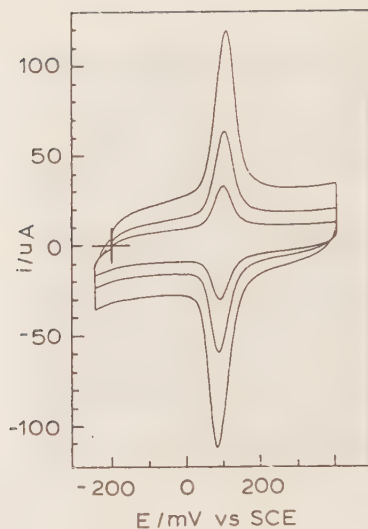


Fig. 3 Cyclic voltammograms of PSCH2 adsorbed on the graphite electrode in a 0.10 M pyrophosphate buffer at pH 7.0, with a surface coverage of 0.95×10^{-9} mole/cm². The starting potential was -200 mV vs SCE and scan rates were 25, 50 and 100 mV/s. $E^{0'}$ = +95 mV vs. SCE.

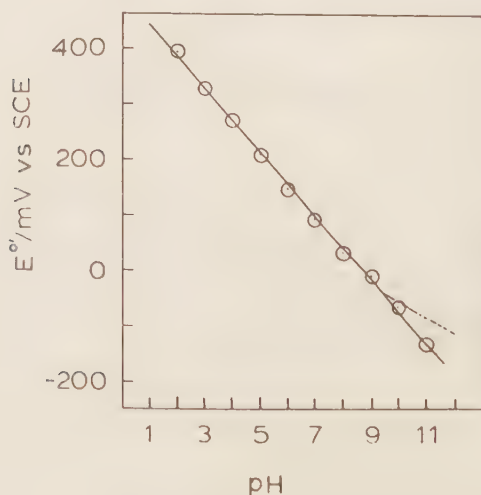


Fig. 4 The $E^{0'}$ for the adsorbed PSCH2 was found to vary linearly between pH 2.0 and 11.0 with a slope of 58 mV/pH. Since the pK_a of pyrocatechol is 9.8, a change of slope would be expected at higher pH values (dotted line). The influence on the pK_a by the pyrene-stilben side chain would be expected to decrease the pK_a , due to the stabilizing effect of the conjugated system to the deprotonated species.

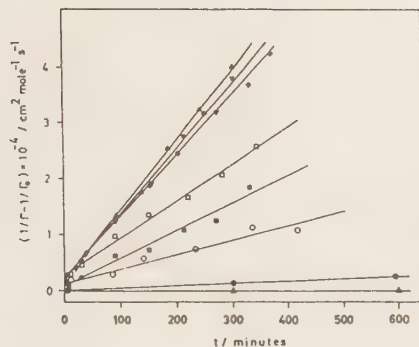


Fig. 5 A second order plot, i.e. $(1/r - 1/r_0)$ vs time, for the decrease of the adsorbed electrochemically active PSCH_2 yields a set of straight lines with different slopes depending on the applied potentials: $\blacktriangle$ 100mV, $\bullet$ 0 mV, $\circ$ 50 mV, $\blacktriangledown$ 90 mV, $\triangle$ 115 mV, $\blacklozenge$ 135 mV, $\square$ 200mV and $\blacksquare$ 250 mV vs SCE. The linearity of the plots as well as the potential dependence of the slopes (Fig. 6) supports the proposed second order chemical deactivation mechanism. See text for further details.

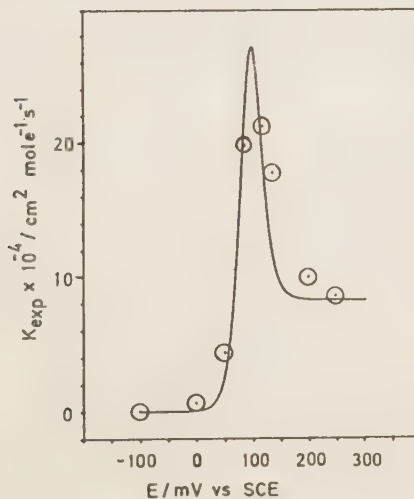


Fig. 6 The figure shows a plot of the observed decay rate, k_{exp} , of electrochemical activity for adsorbed PSCH_2 at different potentials (Fig. 5). The solid line is a theoretical curve using $k_a = 10 \cdot 10^5 \text{ cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$ and $k_b = 0.8 \cdot 10^5 \text{ cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$. See text for further details.

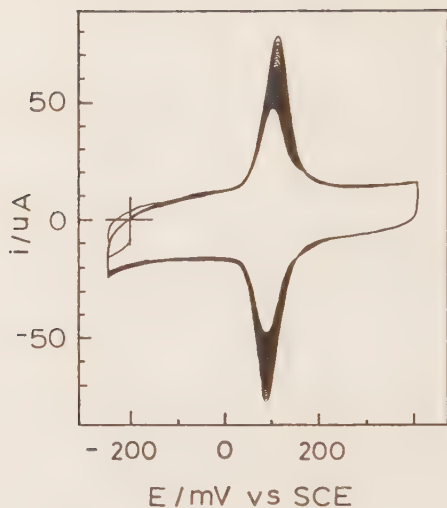


Fig. 7 Cyclic voltammograms of PECH2 adsorbed on the graphite electrode in a 0.10 M pyrophosphate buffer at pH 7.0 with an original coverage of 1.5×10^{-9} mole/cm². The starting potential is -200 mV vs SCE. The scan rate is 50mV/s. $E^{0'} = +100$ mV. A sharp decrease of the electroactivity is seen during the 80 scans shown. Under the same conditions a PSCH2 modified electrode lost only 5% of its electroactivity.

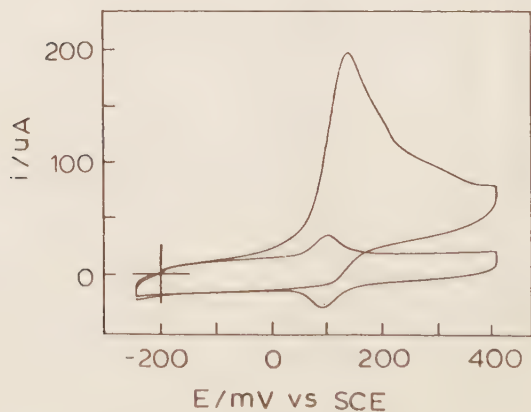


Fig. 8 Catalytic oxidation of NADH mediated by adsorbed PSCH2 (curve A), 2.0 mM NADH, pH 7.0, scan rate 50 mV/s. Curve b shows a PSCH2 modified electrode in a buffer solution without NADH. Coverage 0.4×10^{-9} mole/cm² and starting potential of -200 mV vs SCE.

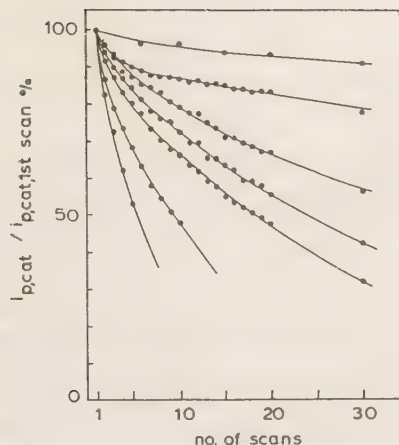


Fig. 9 Plots of $i_{p,cat,1st\ scan}$ versus the number of scans, in a 2.0 mM NADH solution containing in 0.10 M pyrophosphate buffer at pH 7.0. The starting and switching potentials are -200 mV and +430 mV vs. SCE. The scan rate was 50 mV/s. The coverages are 0.10, 0.21, 0.43, 0.56, 0.95, 2.0 and 2.7×10^{-9} mole/cm². The lowest coverage produces the fastest rate of catalytic decay and the highest coverage the slowest. The entire experiment was performed in the same NADH solution for all the electrodes.

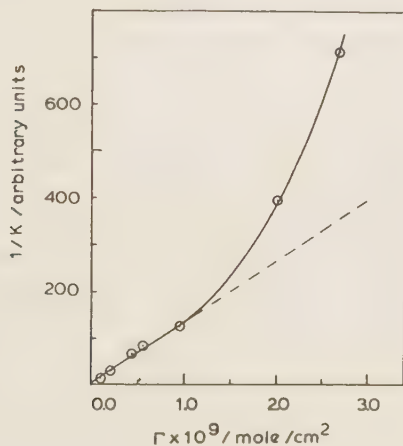


Fig. 10 A plot of the log of $i_{p,cat}/i_{p,cat,1st\ scan}$ versus the number of scans, shows that the catalytic decay (Fig.8) follows the equation $Y = Y_0 \cdot 10^{-(k \cdot (n-1))}$. When the inverse of the slope in this equation (i.e. $1/k$) is plotted versus the coverage of the electrode, a linear relation is found for coverages below 1×10^{-9} mole/cm². A relatively lower catalytic decay rate is observed for the two highest coverages, 2.0×10^{-9} and 2.7×10^{-9} mole/cm².



Fig. 11 The figure is a model depicting how the electrochemically active mediators adsorbed on the electrode (striped ellipses) are blocked from the solution by the bulky NADH molecule after the chemical coupling reaction between the quinone and NADH has occurred. Only the mediator chemically coupled to the NADH molecule is electrochemically inactivated (closed ellipse). The open ellipses represent mediators still available for catalysis.

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